

1/8 January 1981

Signs of grace (and opposite) for 1981

All new years are important. This, 1981, will be crucially so. By next December, it should be clear how several influential governments are tackling the problems which now confront them. For the time being, the most absorbing question is whether Mr Ronald Reagan will live up to his election promises — and threats. Before the year is up, however, there should be a new government in the Soviet Union. Even when the fear that change means danger is as deeply ingrained as in Moscow, it is unthinkable that Mr Brezhnev will be kept on as president much longer. In Western Europe, the chances are that the same old governments will have to battle with the same old problems, but with a sense of mounting despair. If the problems of economic stagnation coupled with inflation appear as obdurate by the end of the year as they seem now, people will begin to behave as if these problems have come to stay. So what are the signs to look for in the months ahead?

Economic problems matter because they determine the resources available for existing, cherished, institutions and for innovation. The industrialized democracies now all suffer to some degree from the disease pioneered first by Italy and then by Britain — rapid inflation born of democratic voters' convictions that living standards must inexorably rise, even when productivity is falling or when resources are transferred in substantial amounts to OPEC oil producers. The consequences are all too clear — industrial investment and research and development are starved of resources, productivity falls still further, the pool of wealth for sharing out diminishes and the dole queues lengthen. One of the first tests of Mr Reagan's temperament in office rather than on the hustings will be the fine print that will no doubt accompany his promised tax-cut. The promise is too fresh in people's minds to be forgotten, but a tax-cut unrequited by reduced federal expenditure would be inflationary. So will the offsetting cuts be intelligent (housing subsidies, pointless environmental protection, excessive solar energy research), stupid (education, research and development) or — horror — non-existent? Other governments in industrialized countries are likely to take their cue from Washington. If Mr Reagan ducks the economic issues, many other governments will know that there is no point in their struggling separately against inflation.

The Soviet economic problem, outwardly different, is as worrying — and not exclusively for Soviet citizens. In the whole Soviet bloc, there is less wealth to share than the managers of the economy would like. Inflation is, by definition, not a problem. Whatever people's hopes, their shares of what is to be shared are determined by the system of allocation. One consequence is the grinding deprivation of much of the Soviet system, partly the cause of troubles such as those with which Poland has recently been beset. The question which the anonymous electors of Mr Brezhnev's successor will have to face is whether the new Soviet government should risk some relaxation of the domestic system for the benefits that would ultimately accrue in improved economic performance. Probably they will shrink from taking such a step on the grounds that the risks of relaxation would be immediate and the benefits delayed. That will be bad news both for citizens of the Soviet bloc and for the rest of us. The needless economic privations of Eastern Europe and beyond are not simply cruel to those who bear the brunt of them but part of the cause of continuing trouble between East and West.

The irony, both in the West and the East, is that the ingredients of continuing technical change and economic improvement are clearly apparent. The cost of petroleum has become a burden, but nuclear power stations are partially a substitute. The new

computer technologies, wildly oversimplified though they may have been, are gathering dust for lack of investment capital and sufficiently skilled manpower. Communications technology, physical and electronic, is held back by lack of capital but also by regulatory bumbledom. Agriculture, surprisingly the great success of the industrialized West in recent decades, languishes elsewhere. The outstanding challenge for most governments in 1981 will be to make a fresh start on using these ingredients of prosperity to enable their people to feed, clothe and house themselves decently, and to have something left over to do what they like with. For many Western governments, but especially for the American, this will require something of a battle with the powerful lobbies which have grown up in the past decade and which appear, after all, to think that there is such a thing as a free lunch — the benefits of technology but no generating plants within sight or the benefits of new drugs but no experiments with animals please. Mr Reagan's reputation suggests that he might throw himself cheerfully into such a fight, but only time will tell.

Two other issues will be bellwethers for the coming year, with policies on defence and arms control most vividly in people's minds. Mr Reagan seems to be committed to winning the arms race, whatever that may mean. Nobody, not even the Soviet Union, will be surprised if the Pentagon's budget now grows again. People in Europe will bite their nails. The danger is that one side or the other will be tempted to check whether it is really winning by pressing all the buttons. The chances are, however, that the arms race of the 1980s will not be very different from that of the 1950s — the modern equivalent of sabre rattling, a diversion of resources from more constructive purposes but adventitiously a source of further technological innovation. What matters much more, especially where Mr Reagan is concerned, is whether he will have the wit and the courage (for a Republican) to seize the opportunity in arms control with which he has been presented. The new administration is committed to reopening Salt II, but Mr Reagan is not the out-and-out abrogator who began the election campaign. As Inauguration Day approaches, he will be made increasingly aware of the advantages, philosophical and political, from balancing his part in the arms race with an open-minded view of what package might replace Salt II. The Comprehensive Test-Ban Treaty, nearly ready for signature though it is, is probably too much of a pill for the Republican Senate and General Alexander Haig to swallow. There is more room for manoeuvre on what are called theatre nuclear weapons — the missiles that must worry the West Germans but also, no doubt, Soviet military planners as well.

A less obvious sign of the way the wind is blowing in 1981 will be the way in which governments, but especially those of industrialized countries, shape in their dealings with the developing world. Mr Reagan's reputation is not encouraging but, to be fair, his position on the recommendations of the Brandt Report (as on many other issues) is not all that explicit. Again, the new president has the appearance of a conservative who may be pushed in liberal directions by events or even arguments. The obvious difficulty is that the months ahead are likely to see still more occasions on which developing countries act uppishly towards the United States (provoking pressure to put them in their place), but that there is no powerful political constituency for the decently self-interested policy of adequate assistance, technical and financial. In the long run, however, there will be no point in beating inflation or surviving the arms race ahead if the world that is left remains as dangerously divided into rich and poor.



Beating a retreat from Rothschild

British governments have long experience of saying one thing and doing another, and the skills thus acquired are now being applied to the management of civil science. Less than ten years have passed since a previous Conservative government (Mr Edward Heath's) embraced Lord Rothschild's then radical notion that government departments seeking benefits from applied research should pay for it out of their own budgets. For most of the 1970s, the British government was devoted to this principle, with the result that government departments (called "customers") and research councils (called "contractors") have been locked in almost perpetual negotiation about the terms on which specific research projects should be carried out. When the Rothschild era began, the research councils affected were deeply offended, and for a time it seemed as if the then president of the Royal Society, Sir Alan Hodgkin, would join them on the barricades. In the event, it seems that the anti-Rothschild forces need not have bothered. They could have counted on the unwillingness of government departments to change their ways.

A few weeks ago, it became clear that the Medical Research Council had won its decade-long battle with the Department of Health, and had won back the share of its budget filched by the department with effect from 1973 (see *Nature* 23 October). Now, the Agricultural Research Council's relationship with its principal "customer", the Ministry of Agriculture, Fisheries and Food, is being changed but in a different direction — the ministry seems likely to have a more direct say in what the council does (see *Nature* 11 December).

This turn of events has an obscure origin — a report of the Public Accounts Committee of the House of Commons published in July 1979 dealing with the cost of storing skimmed milk and potatoes as well as with the affairs of the two research councils. The proceedings, nevertheless, have an arcane interest. Dr James Gowans, the secretary of the Medical Research Council, gave the committee a winsome account of the importance of good luck in the conduct of medical research. The steps in medical research in the past twenty years that "have made the greatest impact. . . have all been made by accident", and as a consequence there is nothing that a research council can do but back "good ideas and good people". Customer-directed research is, in other words, impossible.

The committee, known to the popular newspapers in Britain as the "watchdog" over public expenditure, softened to putty, swallowed the line taken by Dr Gowans and the Department of Health that it was "impracticable" for the ministry to control the research it had commissioned, and recommended that for medical research the government should abandon the Rothschild principle. Both parties have lost no time, and the arrangement has indeed been unscrambled.

With agriculture, similar arguments appear to have led the Public Accounts Committee to the opposite conclusion. The committee seems to have been bamboozled into believing that the Agricultural Research Council supported research intended to yield practical benefits (plant-breeding, for example) with funds left untouched by the Rothschild transfer to the ministry. The committee, evidently ignorant of the council's terms of reference, kept asking why. And why should there have been so little change in the pattern of the council's work since 1972? Surely the only explanation is that the ministry's influence was too remote, and the only possible remedy that the ministry should have a more direct say in what the council does? The ministry has leapt at the chance. It may not be long before the Agricultural Research Council is indistinguishable from a government department.

This is an odd way in which to tackle an issue of principle. The Public Accounts Committee of the House of Commons, for all its virtues, is neither experienced nor competent in fields like this. Its cursory inquiry into the workings of the Rothschild principle was hardly searching enough to give the two ministries the licence that they have so eagerly assumed. The conclusion may be correct that

the Rothschild arrangement has not functioned well in medical and agricultural research, but the explanations given are spurious. Dr Gowans's doctrine of investment in "good ideas and good people" is a necessary but not a sufficient condition for success — and applies in other fields than medical research. And if neither the medical nor the agricultural research programmes have been changed in pattern since 1972, the explanation may simply be the indolence, incompetence or neglect of the ministries concerned. The unhappy tale of how the Department of Health set out to act as a customer by setting up a network of committees whose meetings were as often cancelled as held has been vividly documented in Professor Maurice Kogan's study (with Nancy Korman and Mary Henkel) *Government's commissioning of research* (Department of Government, Brunel University). The wonder is not that the system failed to work, but that its designers thought it might.

Before the rot goes too far, the British government must decide what it is trying to accomplish. Lackadaisical support for supposedly autonomous research councils is demoralizing, and probably wasteful of public money as well. The plain truth is that three of the research councils (agriculture, medicine and natural environment) are geared to basic research relevant to the practice of technology (*pace* physicians). Their autonomy is an historical accident, going back to the First World War and Lord Haldane's doctrine that government departments should not be allowed to control basic research because they would not understand it. Government departments may not be much better now, but times have changed. The Advisory Board for the Research Councils should be the forum in which the research councils make their priorities explicit. In practice, the board seems to be yet another device for making sure that no research council is disappointed in its share of the collective budget. How could it be otherwise, when the chief executives of the councils dominate the proceedings?

The truth is that the five research councils are different animals. The Science Research Council is an essential adjunct of higher education. The Social Science Research Council was set up both to encourage academic social science and to apply the social sciences to the solution of practical problems; it has been only a modest success, but these are early days. All five research councils, together with the Royal Society and the scientific museums, share in the annual cake-cutting ceremonies of the advisory board. (There is even talk that the British Academy, responsible for research in the humanities, should be included.) The result is that the question of how much of the civil research budget should be spent in or on universities, and how much on basic research relevant to technology, is never openly considered.

So what should the British government do? The simple response is to say that there should be yet another inquiry into the organization of civil science. The interval since Rothschild has been longer than any other period since the Second World War when some mammoth reorganization has not taken place. Such a decision, however, would be disastrous. The outcome would be another reorganization which, in the end, would be eroded by the unwillingness of those concerned to change their ways. The solution, which is being forced on the Thatcher government by its unthinking retreat from Rothschild, is a return to the arrangements of the early 1960s, when civil science was the responsibility of a part-time minister. The immediate benefit would be that there would be machinery in being for making policy as the need arose; as things are, nothing is decided between agonized reappraisals. Such a development would have the extra benefit of separating the administration of civil science from that of education (for which Lord Todd was asking in his valedictory anniversary address to the Royal Society in November and will now no doubt be urging on the House of Lords Select Committee on Science and Technology). How much longer will it be before the government recognizes that its neglect of the past few months now predicates such a course?

AAAS plans pressure on education

Dismay at proposed NSF budget cut

Toronto

The American Association for the Advancement of Science (AAAS) has decided to devote a substantial proportion of its efforts over the next decade to reversing what the US Department of Education has recently described as a growing tide of "scientific illiteracy".

Concerned about the growing gap between scientists and technologists on the one hand and the public and congressional decision-makers on the other, the association plans a series of programmes aimed, in particular, at raising the general level of scientific and mathematics education in the nation's primary and secondary schools.

The announcement was made during the AAAS's 147th annual meeting in Toronto this week. On Sunday, the association's board passed a resolution pledging the AAAS to work with its affiliated science and engineering societies to reverse what it called a "damaging decline" of science and engineering education in the United States.

Mr William Carey, the association's executive secretary, later said that the Carter Administration's apparent intent to cut back expenditure on science education by the National Science Foundation (NSF) was "a severe malfunctioning of the bureaucracy".

This cut was occurring, he said, only two months after the NSF and the Education Department had delivered a report to the President which concluded that there had been a 15-year decline in the national commitment to excellence and international leadership in science, mathematics and engineering.

Mr Carey added that as a result of the Administration's decision to absorb cuts in the NSF budget by focusing in particular on its science education programmes, under the terms of the budget proposals which the President will present to Congress next week, science education would be reduced to only 7.5 per cent of NSF's efforts — the lowest figure for 30 years.

What concerns AAAS is not so much the quantity or quality of university science graduates — the department's report found little problem here, and suggested that where there were shortages or deficiencies, these would probably be corrected by market forces — but evidence of declining educational standards in schools.

Dr Allan Bromley, professor of physics at Yale and next year's president of AAAS, said that it had been a "dismal mistake" to

leave things in the hands of "professional educators". He cited, in particular, national surveys of educational achievement which demonstrated a general decline in scientific and mathematical abilities among high school students, as well as widespread evidence of the remedial action which universities and colleges were having to take with undergraduates enrolled in science classes.

"A whole generation of students is being short-changed" Dr Bromley said. "In physics, if you look at undergraduate curricula across the country, the first year is being increasingly devoted to teaching subjects that you would not have had to worry about 10 or 15 years ago."

Other AAAS officers were slightly less harsh in their criticisms. Current president Dr Frederick Mosteller of the Harvard School of Public Health pointed out that many subjects, such as calculus, were now widely taught in schools where they had been missing a generation ago. Retiring president Dr Kenneth Boulding spoke up in support of less conventional teaching methods, arguing that "education is becoming less important as a means of

learning".

However, what Dr Bromley described as a "severe crisis" in science education has spurred the AAAS into convening a conference of the heads of its affiliated societies to look at the health and priority needs of science and engineering education in the United States in the 1980s — and to make educational excellence in these areas a major theme of next year's meeting.

The association also intends to use its new monthly magazine, now called *Science 81*, to produce teaching materials for use in secondary schools, and it announced that Dr F. James Rutherford, previously associate director of NSF responsible for science education, and at present assistant secretary of the Department of Education, will join AAAS as adviser on science education to the board of directors after leaving office on 20 January.

"The AAAS cannot solve the problems on its own, but if we don't get busy, things will get worse" said Dr Mosteller, adding that both federal agencies and private industry would probably be approached to support the association's proposed activities.

David Dickson

Slow progress on Greek reforms

As Greece enters the European Community (from 1 January) the universities are still in the throes of reorganization, with student unrest at some institutions. But the new Science Research and Technology Agency is in good shape, with two years of constructive work behind it, although the threat of its becoming ensnared in Greek bureaucracy remains. The hope is that the influence of the community will strengthen the recent tendency to reform.

The government's first attempt at university reform (see *Nature* 2 February 1978) met with strong opposition and the enforcement of some of the provisions of the new Law 815 had to be postponed. A committee was set up to make new proposals and these have now been presented to the government. The success of the committee, representing the fourteen university-level institutions in Greece, owes much to the way in which its chairman, Professor F. Mitsis, enjoys the confidence of all sides.

Much deadwood was painlessly removed from the academic profession by the provisions in Law 815 for voluntary retirement — in one department of the University of Athens only three out of twelve are still in post. Now there are many openings to be filled using new selection procedures.

The new draft bill would abolish the unpopular system of "chairs", in which professors were surrounded by a mixed bag of assistants. Instead subject "sectors" would be introduced, each consisting of at

least seven academics at three grades: lecturers, associate professors and professors. The short-term appointment of assistants, usually working for higher degrees, would also be allowed. Undergraduates are granted 20 per cent participation in all university bodies under the proposed law.

Examination regulations are being drafted separately, amid protests from student associations that as long as general conditions in the universities leave so much to be desired, students cannot be expected to attain high standards. At the Technological University at Athens there was a prolonged sit-in to protest against the system of semesters, considered unduly onerous by many students. And at the University of Athens chemistry students have been striking against the alleged harshness of a particular professor.

Greece's newest university, in Crete, remains in a critical state. Facilities are makeshift and most of the teaching depends on visiting lecturers from abroad. Permanent senior appointments are being blocked by the Athens-based administration committee, perhaps under the influence of the old establishment. Fotis Kafatos of Harvard is no longer a member.

For research, the outlook is more cheerful. More than two years ago the government invited Dr George Argyropoulos back from the United States to head the new Science Research and Technology Agency (see *Nature* 23 February 1978). The main achievement of the agency has been the introduction of

project grants on the pattern of the British research councils, instead of state subsidies for the overall budgets of institutions.

Following wide consultations, the agency prepared a list of nine priority areas of research, approved by a ministerial committee in June 1979. By October 1980 436 grant applications had been received and some 70 approved projects were already under way. Applications are being dealt with speedily, the process taking an average of seven months including the time required for three referees (at least one from abroad) to report.

Other achievements include eighteen international agreements for joint projects. Solar energy plants are to be built with Germany and France, and a geothermal plant constructed on the island of Melos in cooperation with the European Economic Community.

There are some clouds on the horizon, however. The law setting up the Science Research and Technology Agency sought to exempt it from the notoriously bureaucratic public accounts regulations, but the Ministry of Finance nevertheless managed to block payments of grants for "technical reasons". The Committee of Ministers which oversees the work of the agency has reaffirmed its confidence in it. One hopes that it will now be allowed to function unhindered.

E. M. Pantelouris

UK space policy

Year of decision

Last year was a busy one for makers of British space policy. By November, a committee of the Central Policy Review Staff, the think tank, had submitted to the Cabinet its study of Britain's efforts in space applications; and an inter-departmental committee, under the Department of Industry, had been created to coordinate space policy more effectively and to discuss the think tank's deliberations. The Home Office was also busy preparing a report on direct broadcast television by satellite.

The sudden interest in space seems to have been stimulated by the fear that Britain might miss out on the profits that could be made from selling space technologies, especially telecommunications satellites. The think tank's report is to remain confidential for commercial reasons. The gist of the recommendations is that there is a demand for space applications satellites which British industry should be encouraged to meet.

One important question is whether Britain should try to build up its industry alone or whether it should continue, perhaps at an increased level, in the space applications programmes of the European Space Agency (ESA) which France and Germany, in particular, have used more effectively than Britain to boost their own industries. The most likely outcome is that Britain will continue at more or less the same level in ESA's telecommunications

programmes, but that greater efforts will be made to transfer the results of ESA's research and development to industry.

The government is unlikely to rush to pour money into the industry, seeking rather to encourage private investment. A central issue in the telecommunications field will be the government's attitude to the monopolies held in television broadcasting by the broadcasting authorities and in satellite communications by the telecommunications division of the Post Office, British Telecom. A bill to dilute British Telecom's monopoly is now before Parliament, and could be used by the Department of Industry to liberalize access to satellite communications, leaving the way open for private operators, in particular, of small satellites for business communications. If greater incentives are given to satellite operators, the next question will be the ability of British Aerospace (which not everyone is convinced could withstand open competition) and other UK manufacturers of satellite components to meet the demand.

A government announcement on the subject is expected soon and it may seem rather bland, leaving the question of monopolies at least until the Home Office has reported on direct broadcasting by satellite. A decision will have to be taken fairly shortly, however, on the scale of Britain's effort in another space application — remote sensing — if it is not to miss the opportunity of cooperating in ESA's next programme. This year promises to be a vital one for Britain's space industry.

Judy Redfearn

Herbicide safety

Bill of health

The herbicide 2,4,5-T has been given a cautious but clean bill of health by two recently published reports. One*, by the Advisory Committee on Pesticides of the UK Ministry of Agriculture, Fisheries and Food, says that there is no sound medical or scientific evidence that herbicides based on 2,4,5-T are harmful to humans, animals or the environment in general. The second, by the European Community's Advisory Committee for Safety, Hygiene and Health Protection at Work, says there is no conclusive evidence that 2,4,5-T causes cancer, but asks for further evaluation of the long-term risks.

The British pesticides committee, essentially the licensing body for pesticides and herbicides, has reviewed 2,4,5-T nine times since 1970. Its latest review was undertaken at the request of the Minister of Agriculture after the National Union of Agricultural and Allied Workers claimed,

*Further review of the safety for use in the UK of the herbicide 2,4,5-T. Available free of charge from Pesticides Branch, Ministry of Agriculture, Fisheries and Food, Room 678, Great Westminster House, Horseferry Road, London SW1P 2AE, UK.

in March 1980, that 2,4,5-T was harmful.

The union reviewed the scientific literature on 2,4,5-T and referred to 20 cases where it was alleged to have harmed humans or farm animals. The advisory committee says, however, that the union's evidence does not indicate that 2,4,5-T is a health risk.

Concern about 2,4,5-T has centred mainly on the presence of a contaminant, 2,3,7,8-tetrachlorodibenzodioxin (dioxin), a teratogen and carcinogen in some animal species. The committee now believes that this concern may have been misplaced and that the risks posed "by dioxin contamination in 2,4,5-T formulations may hitherto have been overestimated".

First, the committee says, dioxin contamination of 2,4,5-T formulations sold in the United Kingdom is now at the low level of 0.01 p.p.m. Second, the committee says that new studies enable it to identify for the first time "a daily level of intake below which effects on reproduction do not occur in the rat", that is 0.001 $\mu\text{g kg}^{-1} \text{ day}^{-1}$.

In the circumstances, the committee considers that 2,4,5-T itself would present a problem before its dioxin contaminant, but that there is no convincing evidence that any effects caused by 2,4,5-T will be passed on to succeeding generations "on an heritable basis". The committee also notes that the WHO/FAO authorities have set a "no effect level" for 2,4,5-T in animals at 3 mg kg^{-1} . Employing a thousandfold safety margin, WHO/FAO have set a "temporary acceptable daily intake" for a man at 3 $\mu\text{g kg}^{-1}$ for 2,4,5-T containing 0.1 p.p.m. dioxin.

The committee does, however, accept the union's claim that workers using 2,4,5-T are not always adequately protected. It suggests that exposure to the herbicide should in future be measured in urinary excretion.

On the question of alternatives to 2,4,5-T, the committee is doubtful. Much less is known about their toxicity and the committee, in continuing to allow 2,4,5-T to be used, is relying on the maxim "Better the devil you know . . .".

All of the twenty cases where 2,4,5-T exposure is alleged to have caused health problems are discussed in the report. The advisory committee has harsh things to say about coverage of alleged 2,4,5-T incidents and accuses the press of causing needless distress by publicizing cases without the consent of the individuals involved. The committee may have been unwise in making this accusation, given that most of the individuals referred to in the report seem to have sought out journalists.

Although the committee has given 2,4,5-T herbicides a clean bill of health, it does ask for prospective epidemiological studies of the risk from exposure to herbicides in general. Professor Robert Kilpatrick, chairman of the advisory committee and dean of the school of medicine at the University of Leicester,

says that a prospective study is in the offing and that 2,4,5-T would almost certainly be one of the herbicides investigated.

The report ducks one issue, however, that of trade union involvement in its decisions. The unions would prefer the Health and Safety Executive (HSE) — where the unions are included on various committees — to be responsible for assessing herbicide safety. Kilpatrick says that this is a matter for ministers to decide. But it seems likely that the unions will be involved in planning the prospective epidemiological study on herbicides. It remains to be seen, however, whether participation on this *ad hoc* basis will satisfy them. Despite the reassuring conclusions of the advisory committee's report, the recent decision by the Trades Union Congress to instruct its members not to handle any 2,4,5-T imported into Britain stands for the time being.

Alastair Hay

UK forestry policy

Research wanted

British policy on forestry research came in for a drubbing just before Christmas in a critical report from the House of Lords Select Committee on Science and Technology. And the annual report of the Forestry Commission, the public agency responsible for publicly-owned forests, did little to answer the criticisms when it appeared a few days later.

The select committee's chief complaint is that there is no coordinated policy on forestry research. The Forestry Commission is accused of being too concerned with wood production to bother promoting less obvious areas of research, so that the Natural Environment Research Council has been left to support "fundamental and strategic research" — a responsibility which it has shouldered "lightly", according to the committee.

The committee asks that the Forestry Commission should mend its ways by appointing a chief scientist, and that the Advisory Board for the Research Councils should decide which of the two obvious candidates — the Natural Environment Research Council and the Agricultural Research Council — should be responsible for longer-term research. The commission should also be urged to provide advisory services for the owners of private woodlands much in the spirit in which the Agricultural Advisory Services help private farmers to tackle agricultural problems.

The commission's own account of the past year (to March 1980) confirms a pre-occupation with the production of wood and in this respect some of the results are impressive. Machinery and pesticides have made the planting of trees a more certain business, with obvious benefits to production costs. The development of local seed supplies, the search for more productive species and for the avoidance of damage by high winds and the control of

Lords look ahead

The House of Lords Select Committee on Science and Technology will study scientific advice to government in its next inquiry. The idea came from Lord Todd, chairman of the committee. The committee will consider whether the post of chief scientific adviser to the government, which was abandoned in 1974, should be reinstated and whether responsibility for science should be removed from the Department of Education and Science.

pests and forest diseases remain the mainstays of the commission's research.

British forestry is a controversial business with the commission frequently pilloried for its preoccupation with conifer plantations. The select committee makes a case for the preservation and renewal of often ancient broad-leaved woodlands, suggesting local authorities as suitable custodians. There is a plea for research to throw light on the more effective planning of land use.

The implementation of many of the select committee's recommendations will cost money — both for reorganization and for an increase in volume of some areas of research. The current level of forestry research funding of just under £5 million for the past year is not generous when compared with the current value of the forest estate of £500 million. But recognizing that an increase in public funds is unlikely, the committee suggests that extra revenue could be created by a 0.1 per cent levy on imported timber and wood products, raising £2.75 million a year.

There is little prospect that extra funds could be found in the Forestry Commission's profits. The commission acknowledges that over the next three years it is unlikely to make the financial target of 3 per cent a year in real terms required of it by the British government. In this respect, the Forestry Commission has yet to make an effective response to the criticism of its financial operations by the Treasury in 1972.

Judy Redfearn

UK clinical research

Future not so rosy

Celebrating their fiftieth anniversary in London last month, members of the Medical Research Society were not altogether optimistic about the future. One of the principal fears, aired at the day-long symposium on the organization of medical research, was the declining ability of clinical departments to attract and train the people to keep them in the front line of research.

Part of the problem is to provide the money and status to keep non-clinical research workers in clinical laboratories, where their knowledge and expertise is essential. Dr J. L. Gowans, secretary of the Medical Research Council (MRC) pointed out that although MRC grants can sometimes cover the costs of such people, the universities should really be providing them with permanent jobs.

Professor W. S. Peart of St Mary's Hospital Medical School, London, said that in hard times, hard choices must be made in medical schools between technical staff and academic staff — it may be necessary to take on a new postdoctoral fellow rather than a lecturer.

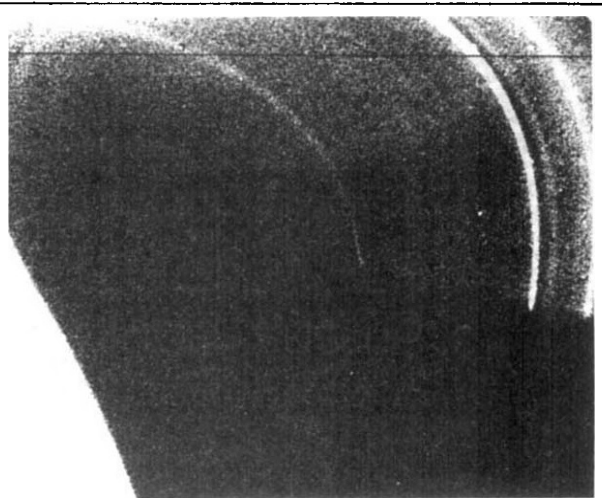
The other side of the problem is the difficulty of providing high quality scientific training for medically qualified staff without disrupting the smooth course of their clinical careers. Professor Peart was disturbed by the lack of young people entering academic clinical research.

The continuing revolution in biology will increase the need to unite basic and applied research on man. Dr S. Brenner, director of the MRC Laboratory of Molecular Biology in Cambridge, pointed out that human molecular and cell biology, now in its infancy, will become the essential interface. In twenty-five years, he said, it will be as essential for researchers in pathology, cardiology and so on to have a thorough knowledge of cell and molecular biology as it is now for clinical endocrinologists to know their steroid chemistry.

Mary Lindley

New rings for old

Claims from ground-based astronomers that Saturn has an innermost D ring, stretching from the bright C ring down to the planet's cloud tops, were apparently disproved by Pioneer 11 in 1979. But this image from Voyager 1, taken soon after its encounter with Saturn last November, reveals an inner ring, complete with fine structure, between the planet (bottom left) and the C ring (top right). Here, Saturn's shadow falls across the ring, which is apparently too faint to be detectable from Earth.



UK universities

Feeling the pinch

Figures released by the Committee of Vice-Chancellors and Principals show that the number of overseas undergraduate and postgraduate students admitted to British universities in October 1980 was 90.7 per cent and 88.9 per cent respectively of the number admitted in October 1979. The intake of British undergraduates was 2.3 per cent higher than in 1979, an increase in line with the rise in the number of 18-year-olds in the population.

The shortfall in the number of overseas students was not as great as some had expected after the British government's decision earlier in the year to charge them the full economic fee for their education, averaging about £3,000 per year. But recent figures released by the Universities Central Council on Admissions (UCCA) indicate that a much greater shortfall may be expected this year. The number of applicants from overseas for admission in October 1981 is 33 per cent down on the

number received at the same stage last year. One reason for the delayed reaction may be that governments had already committed themselves to paying for students to study in Britain this year before the increased charges were announced. Applications from British students for university places in 1981, however, continue to show a small increase, the number from men being up by 2 per cent and from women by 7 per cent.

Figures released at such an early stage in the academic year are not a reliable guide of what will happen next year. Nevertheless, many universities are worried. They will face a difficult decision in February and March when they have to fix their fees for 1981-82. With a considerably reduced income from overseas students, they will have to decide whether to compensate by increasing the fees of overseas students yet again at the risk of discouraging even more students from accepting places.

According to the vice-chancellors' committee, the increase in the number of home students only adds to the universities' problems because the government has set

aside no extra funds to meet the growth. Well qualified home students could well be refused places because of a lack of cash to support them. The full effects of the increase in overseas students' fees will be felt in 1982-83 by which time government protection of overseas students who began their studies before October 1980 will have ceased. The government will then have effectively removed 10 per cent of universities' income from the recurrent grant.

The Committee of Directors of Polytechnics reports a similar but more marked trend for entry to full-time and sandwich courses in October 1980. Admissions of overseas students were down 29 per cent on 1979, but this was more than compensated for by a 7 per cent increase in home students, much of the growth being in science and technology courses.

Some polytechnics with a high percentage of overseas students, especially those near London, may be hit particularly badly. Others may only be able to offer those courses for which there is a buoyant demand. At Portsmouth Polytechnic, for example, the masters' course in fuel technology, the only one of its kind offered in Britain, is very popular. But courses offered by several institutions may suffer badly from increased competition. polytechnics' advantage may, however, be that they can adapt their courses to changing demands more easily than the universities.

Few universities and polytechnics have yet analysed this year's intake in terms of subject area and students' nationalities. But general impressions are that students from South-East Asia are having more difficulty in raising fees than those from the Middle East and that science and engineering courses are suffering less than courses in the humanities.

Judy Redfearn

Brazil's space programme

Aiming high

São Paulo, December

Showing refreshing optimism at a time of unprecedented inflation, plans have been drawn up in Brazil for an ambitious space programme costing hundreds of millions of dollars over the next ten years. Remote sensing and meteorological satellites and the rockets to launch them are included in a plan approved in principle by the Brazilian government.

Meteorology and remote sensing have already attracted substantial interest and investment in Brazil, where the information they can provide on the physical conditions of little-known areas is especially valuable. The remote sensing programme, set up in the 1960s, was intended to help map the Amazon Basin. The programme has grown from the use of aerial survey to using Skylab and Landsat images. A Landsat receiving station was

Poland freezes Antarctic research

Poland's Antarctic research programme for 1981 will include a "wide range of economy measures" according to a statement last month by Dr Maciej Zalewski, Secretary of the Polar Research Committee of the Polish Academy of Sciences. From now on, he said, research will concentrate on "strategic lines" of special importance to science and the economy.

The main saving, said Dr Zalewski, will be made by discontinuing large and expensive summer expeditions, and concentrating on small teams occupying a permanent base on a yearly shift system. Nevertheless, some summer activity will

continue — such as the current joint expedition from the Academy of Sciences and the Marine Fisheries' Institute in Gdynia which is part of the international "Biomass" programme, as is the similar expedition planned for summer 1982-83.

What, then, is to be cut? According to Dr Stanislaw Rakusa-Suszczewski, of the academy's Institute of Ecology, very little of any significance. Antarctic programmes are planned by the three relevant institutes of the academy — geophysics, biology and ecology — and financed by the academy, and judging the proposed research entirely on its intrinsic merits, the arguments were so strong, he said, that the academy has decided to go ahead. Under the new programme, the main emphasis will be on marine biology and the study of whales, but in all disciplines there would be increased stress on fund a mental research.

This represents a significant change in research planning. Since the early 1970s, research in Poland has been target-oriented and financed on a project basis, within a complex hierarchy of "problems" of the national economy. (Fundamental research was still possible, but had to be formally associated with some specific "problem"). The new intellectual climate in Poland, with its emphasis on greater academic autonomy, described by Dr Rakusa-Suszczewski as a "very positive change", has clearly meant that, in reshaping the Antarctic programme to fit Poland's reduced economic circumstances, the planners of the academy have been able to make their decisions on basis of scientific value.

Vera Rich



Arctowski Station, King George Island.

opened in Curitiba in 1975 and now provides 20,000 images a year to 1,100 users, many of them in other South American countries. The next step will be to upgrade the station, at a cost of about \$6 million, to receive Landsat D images.

The meteorology programme involves the building of up to 100 data-collection platforms around the country. A prototype is being tried out in France and the first Brazilian platforms are expected to start operating early this year. Information from them will be relayed by the American TIROS N and GOES satellites. A major objective of the platforms is to provide accurate forecasts of drought in the impoverished north-east region.

According to Dr Jesus Parada, director of the civilian space research institute, INPE, previous cooperative ventures, mainly with America, have given Brazil the necessary expertise to go it alone.

The intention is to launch four satellites a year from 1986–87. INPE would develop the satellites and the launcher would be developed at the nearby military space institute. Total cost of the programme is estimated at more than \$700 million, about two thirds of it for launcher development.

The first two satellites would take over from TIROS N and GOES by relaying information gathered by the meteorological data collection platforms to a national centre. And the number of platforms could be increased to several thousands if the new satellites are launched. A radiation budget experiment is also planned. The first satellite, weighing 150–200 kg, would be launched on the qualification flight of the rocket into an orbit of 25–30° at an altitude of 700 km.

The third and fourth satellites would be more complex, devoted to direct land imaging and weighing 250–300 kg. They would be placed in a near-polar orbit at an altitude of 650 km.

All four satellites would be launched from a site in Brazil, possibly the existing military launch pad near Natal. A major drawback of this site, however, is that large payloads would have to be launched into an unusual trajectory to minimize the threat to the city which is only a few kilometres away.

Ambitious though the new plans are, progress so far has been slow. With a budget of only \$10 million for 1980, feasibility studies have not got as far as Dr Parada would have liked. But if the hoped-for budget of \$40 million is forthcoming for 1981 the project can move on into the design phase.

In a country with grave social problems there are many areas where investment might provide a more obvious and immediate return than a venture into space. And a second space project, for Brazil's first telecommunications satellite, is also competing for funds. So the chances of Brazil's space programme are closely tied to the fate of Brazil's economy over the next few years.

Judy Redfearn

Nuclear waste

US Senate stalls

Washington

Hopes for new legislation establishing a national policy for the disposal of radioactive waste — eagerly sought by both the nuclear industry and the environmental movement — foundered in the Senate last month on disagreement over whether states should be involved in decisions about military waste from nuclear weapons construction.

Main responsibility seems to rest with Senator Henry Jackson, powerful chairman of the Senate Energy committee and a member of its Armed Services Committee.

Senator Jackson had previously supported a proposed nuclear waste bill that was passed in the Senate in July. This bill contained limited provisions for the involvement of states in siting decisions, which the Senator opposes, but it was sweetened by the inclusion of plans for "away-from-reactor" sites which the owners of nuclear reactors could use for the temporary relief of their own spent-fuel storage tanks.

The House of Representatives, after lengthy negotiations between pro-nuclear and anti-nuclear forces, passed a compromise bill last month. Like the Senate bill, it provided for a state to veto a siting decision, if it obtained support of one of the two congressional bodies.

However, the House bill did not include any mention of away-from-reactor storage, which the industry would like since it would keep spent fuel available for future reprocessing. And Senator Jackson made it clear that he did not support the bill, killing any chance of agreement by offering an amended version unacceptable to the House.

The storage of nuclear waste remains the Achilles' heel of the nuclear power industry, with several states currently refusing permission for further power-plant construction until adequate means for long-term storage are available.

The nuclear industry itself is keen to find more permanent storage for the 80,000 metric tons of spent fuel now lying in storage tanks next to reactors around the country. And the environmental movement wants a nuclear waste policy that is both ecologically acceptable and open as much as possible to local participation in decision-making.

In February, President Carter announced plans for a long-term waste disposal policy. Reiterating his previous opposition to reprocessing, this is based on the permanent storage of waste in deep geological formations — a technique considered both feasible and relatively safe by most of the scientific community.

Carter proposed that long-term studies of possible sites should be initiated, with the goal of having a permanent disposal facility in operation by 1995. But the

Senate, acting under pressure from the nuclear industry, expressed impatience with this schedule, and the bill which it passed in July emphasized short-term waste storage procedures.

Sharp differences also occurred in the House of Representatives. Pro-nuclear forces, led by Congressman Mike McCormack of the Science and Technology Committee, supported a bill which would require the Department of Energy to construct technology demonstration facilities in various parts of the country, to serve as a basis for licensing a full-scale commercial waste facility.

In contrast, the chairman of the House Interior Committee, Congressman Morris Udall, supported a bill more closely modelled on the Administration's proposals, arguing that it was premature to rush into demonstration projects.

In the end a compromise was reached between the two committees and the House passed a joint bill, under which the Department of Energy would pick two possible burial sites by 1982 and two more by 1985; and by 1987, following an elaborate series of public hearings, the President would recommend to Congress the site or sites which he considered safe for the storage of waste.

Despite the faster schedule, this compromise proved unacceptable to the Senate. Stung by the omission of any provision for short-term away-from-reactor storage, Senator Jackson told the Senate that without a specific exemption for atomic defence repositories, the House bill as it stood infringed the jurisdiction of both House and Senate Armed Services Committees, and could conflict with existing clauses in the Atomic Energy Act.

Environmental groups are insistent that military wastes, amounting to up to 90 per cent of the nation's high-level radioactive waste, should also be covered in any national plan.

But the Senate's decision now means that the process of developing legislation must start over again when the new Congress convenes this month. In the Senate at least, recently-elected conservative legislators are likely to be more sympathetic to the nuclear industry.

Meanwhile, in line with the Carter Administration's long-term waste disposal policy, the Department of Energy has issued an environmental impact statement confirming its view that the best method of permanently disposing of radioactive wastes from nuclear power plants is to place them in mined depositories deep in geological formations.

According to the department, a typical repository would require about 2,000 acres underground, and above-ground facilities would occupy 500 to 750 acres. Access roads would take up another 30 acres. A total of 2,000 acres above ground would be needed for each facility, with consequent restrictions of mineral and surface rights.

David Dickson

CORRESPONDENCE

Badgers and TB

SIR — I refer to the letter by Dr Stephen Harris in your issue of 11 December 1980 (p.532) which, while critical of certain aspects of Lord Zuckerman's Report to the Minister of Agriculture on "Badgers, Cattle and Tuberculosis", agreed with his basic conclusion that the badger is a major reservoir of bovine tuberculosis (TB) in some areas of South-West England. The main criticism was that, while the reservoir constitutes a potential danger to cattle in limited areas, there is no "scientific evidence" to justify the deduction that the level of tuberculous infection in badgers in the South West is "dangerously high" and may result in spread of the disease to adjacent populations.

Since Dr Harris writes for and on behalf of the Mammal Society, it may be appropriate to ask whether all the interested members of that society would fail to be convinced by the evidence. More than 4,000 badgers were examined in connection with official investigations in the years 1971–79 and 14 per cent at least harboured *Mycobacterium bovis* (Report: para. 130). Of animals found dead since 1972 in fields, woods and farms in Gloucestershire, Avon and Wiltshire, 54/194 (28 per cent) were found to be tuberculous. Of those killed in Gloucestershire, predominantly on the roads and presumably by chance, during this period 1976–80, 21/232 (9 per cent) were tuberculous; this admittedly was the highest recorded prevalence by county but for the whole of the South West the figure was still 4.2 per cent (Report; Table 3).

These figures should be a cause for concern when dealing with a social animal which occupies, for much of the time, confined spaces underground and whose movements above ground favour between-group contacts. Bovine tuberculosis in badgers is sometimes a rapidly progressive, lethal disease, the causal organism probably being excreted by many routes in large numbers^{1,2}. It has, therefore, ample opportunities for transfer and by any objective epidemiological standard the risk of spread to neighbouring badger populations must be considerable. The onus for showing that this is not a reasonable interpretation should rest on others.

The argument advanced by Dr Harris, that the recent decline in the incidence of tuberculosis in cattle in the South West is simply a reflection of a general downward trend in the figures for England as a whole, misses the point that the incidence of herd "breakdowns" in the South West, in the years 1974–80, has never been less than five times that for the rest of England (Report; Table 19).

Many anomalies in the data were highlighted in the Report, which pointed out

the need for continuing investigation and research. In this connection it is difficult to see how the "subtle factors" twice mentioned by Dr Harris as significant in the spread of the disease from infected badgers, could be elucidated without extensive sampling on a statistically significant scale. In particular, continuous monitoring of badgers on the perimeter of heavily infected areas would be necessary to prove that the disease is *not* spreading (Report; para. 137). It is to be hoped that consideration will be given by interested bodies as to how this could be organized, if the necessary funds were provided.

W. PLOWRIGHT

Department of Microbiology,
ARC Institute for
Research on Animal Diseases,
Compton, Berkshire, UK

1. Gallagher, J., Muirhead, R.H. & Burn, K.J. *Vet. Rec.* **98**, 9-14 (1976).
2. Gallagher, J. & Nelson, J. *Vet. Rec.* **105**, 546-551 (1979).

Museum policy

SIR—The broader implications of cladism are not my special concern, but the study of human evolution is. Cladistics is a useful research tool, but I support L.B. Halstead's¹ objections to the way it is used in the new exhibit *Man's place in evolution* at the Natural History Museum.

Cladistic analysis is still a relatively untried method for expressing relationships between groups, and technical details of its application are still being debated. It was devised as a conceptual framework within which one aspect of the relationships between modern and fossil forms could be analysed. It relates groups solely on the basis of shared features, and rejects the notion that any ancestral relationship can be inferred from this evidence; such relationships are considered "unknowable". Proponents claim that only by adhering to these principles can palaeontologists frame testable hypotheses. The strengths of the method are its simplistic rigour, but therein also lie its weaknesses. In order to provide an unambiguous framework, certain assumptions have to be made. For example, speciation is recognized only as a dichotomous branching event, and more complex adaptive radiations, anagenesis and convergent evolution of morphological features are discounted. Such assumptions are unrealistic. The concept of "relatedness" enshrined in cladistics is therefore fundamentally different from the general one which implies a lineal relationship, and as used in the exhibit, it is likely to confuse, if not actually mislead, the visitors.

The contents of the exhibit also give cause for disquiet. The authors seem particularly confused about what constitutes the "habiline" and "australopithecine" groups of early hominids. Two important specimens "1470" and KNM-ER 1813, have been seriously misassigned. KNM-ER 1813 is consistently cited as an example of a "habiline" and "1470" as an "australopithecine". Yet, "1470" has a cranial capacity of around 775 cm³ and is included in *Homo habilis* by many workers.

The cranium KNM-ER 1813 is smaller overall, and it has a cranial capacity of between 500 and 550 cm³. It has not yet been formally assigned to a taxon but its position is sufficiently enigmatic for it to have been compared, by some authors, to *Australopithecus africanus*, and by others, to early *Homo*. Howell² has attributed KNM-ER 1813 to *Homo habilis*, but he also includes "1470" in the same species.

The section which deals with *Homo erectus* also deserves comment. Recent, and well known, discoveries in Europe and Africa have demonstrated that a much wider range of cranial morphological features is subsumed into the "erectus" group than was previously believed. Several skulls shown an apparent mixture of classic "erectus" and archaic *Homo sapiens* features. These new finds strongly suggest a continuous gradation of morphology from "erectus" to "sapiens". The exhibit totally ignores this evidence.

To attribute important finds wrongly is careless; to ignore "uncomfortable" evidence is dubious academic practice, and to imply that cladism is an orthodox systematic approach is irresponsible. The contribution of the Natural History Museum to science and education is too important to be compromised in this way.

B.A. WOOD

Middlesex Hospital Medical School,
London W1, UK

1. Halstead, L.B. *Nature* **288**, 208 (1980).
2. Howell, F.C. in *Evolution of African Mammals* (eds Maglio, V.J. & Cooke, H.B.S.) 154-248 (Harvard University Press, Cambridge, 1978).

SIR — In a recent letter to *Nature* (288, 208; 1980) L. B. Halstead states his convictions that human evolution was gradual, that its gene pool of the past had certain knowable characteristics, and that the ancestry of a living species can be determined with deadly accuracy. Halstead's convictions arise from his discovery of a new form of *doubt*, such that he can contend that "there is not any serious doubts about *Homo erectus* being directly ancestral to *Homo sapiens*". This is certainly the news Biology has waited for, the moment when the Truth can at last be known so that all this difficult and extremely tiresome theory can be dispensed with. Until now my colleagues and I had always imagined that to doubt something was "to be uncertain as to a truth or fact" and the notion that distinguishes science from, say, politics is that in science uncertainty about the truth must remain or progress ends. Halstead's discovery can only mean that he has in hand a new form of truth — a kind of truth that can be known. Many of us puzzled over what kind it might be until it finally dawned on us that it emerges from false doubt and, to honour its discoverer, I call it *Halsteadian Truth*. If ever again we are troubled by some theoretical matter we need only consult the oracle in Reading. Now that Halsteadian Truth permits us to know at last the way in which evolution proceeds, what extinct gene pools were like, and exactly who was whose ancestor, I confess, with sadness for years misspent, that cladistics is indeed a waste of time.

This news about the emptiness of cladistics will have a profound effect on systematics, for

Continued on p. 105

From the Mammal Society:

In the letter from the Mammal Society on badgers and TB published on 11 December, the statements appear to be attributed to Stephen Harris. I wish to make it clear that the letter is a Mammal Society view and that Stephen Harris was only personally involved in the communication of the letter to the Editor.

— J. R. Flowerdew, Hon. Secretary.

NEWS AND VIEWS

The ocean floor closely observed: will it subvert marine geology?

from Tjeerd H. van Andel

In a recent paper based on work with a deep-diving research submarine, fine-scale observations of the sea floor are ingeniously extrapolated to models of the events occurring at depth*. The paper is a good example of the geologist's method of using detailed observations on rock outcrops to yield grand interpretations of causes, and it is as such quite similar to the usual fare in geological journals except for an important difference — the observations were made 2,000 m below the sea surface rather than on a walk in the hills.

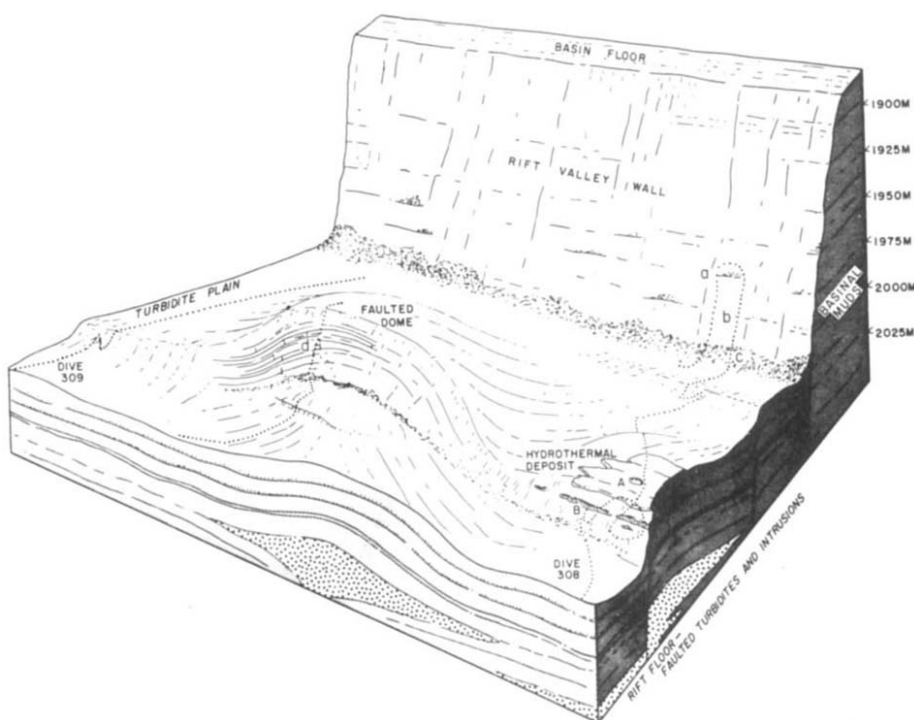
For a century, marine geologists have coped with severe limitations imposed by the vastness of their domain, by the filter applied to their observations by water depths of several kilometres, and by the difficulty of knowing where one is. The first of the constraints produced descriptions of entire ocean basins equivalent to a geological synthesis of Britain based on ten outcrops, while the second and third limited resolution to features tens or even hundreds of metres across. The traditional oceanic mapping scale is about 1:1,000,000, and in the 1960s a 'detailed' map was usually drawn to a scale of 1:250,000. We did, of course photograph the sea floor but the gap between a photo of a few square metres and a map representing the terrain at a scale of 2.5 km to the centimetre is near unbridgeable. We observed mid-ocean ridges and sand ripples but very little in between. Until a decade ago, the divergent plate boundary appeared as a V-shaped cleft on sounding records; now we know that it consists of vertical fault scarps, terraces, flat lava floors, rows of small but complex volcanoes and fascinating hot springs.

About a decade ago our ability to

appreciate detail improved rather suddenly with the introduction of instrument vehicles towed near the ocean floor, manned submarines and, above all, with the means to determine positions within ten metres or so. Since 1970, large expeditions to several mid-ocean ridges have demonstrated the power of the new technology with numerous startling discoveries, and have introduced new ways of thinking about marine geology that are akin to traditional approaches in petrology and structural and sedimentary geology on land. At times the change has been disconcerting to marine geologists not

accustomed to looking at their world on a fine scale, but the new opportunities clearly hold much promise.

The impact of this costly and logistically complex technology has not been restricted to scientific data and inferences. There has been some sense of discouragement. A decade of hard work has covered little territory at a high cost. Perhaps 0.01 per cent of the divergent plate boundary has now been studied in detail at a cost of much more than 10 million dollars. One wonders whether, at this rate, we shall ever be able to obtain a reasonable sample of the rest of the ocean floor. The work is also



A sketch of the undersea terrain near to the north west wall of Northern Trough, Guayanas Basin, Gulf of California from two dives made by Lonsdale and Lawver in the submersible *Sea Cliff*. The Gulf lies between two crustal plates that are drifting apart, in a position like that of the Mid-Atlantic Ridge between the American and the European and African plates. At mid-ocean ridges, as the plates separate, molten rock wells up from below to fill the rift and heal it, forming a string of small volcanoes. In the Gulf of California the same process occurs but is modified by a thick blanket of sediments shed from the nearby Continental margins. As a result the lava does not reach the surface but forms mushroom shaped intrusions within the sediment layer and deforms and domes the overlying deposits and sea floor.

*Lonsdale, Peter, and Lawver, L.A. *Immature plate boundary zones studied with a submersible in the Gulf of California*; *Geol. Soc. America Bull.*, 91, 1, 555 (1980).

Tjeerd H. van Andel is Professor of Oceanography in the Geology and Geophysics Departments of Stanford University, California.

cumbersome and improving the instrumentation is sometimes more challenging than using it. This is not without reason. The paper that provoked this discussion, based on minimal technology, suffers the weakness of a consequently much reduced precision.

Coupled with this image of an overwhelming task is a feeling of insecurity. Does any value, except crude geographic description, remain in the work done before? Are the sparse observational nets, the data of low resolution still useful? Does the image of the ocean floor obtained over the last century still hold up? Of course no one was ever wholly unaware of the shortcomings of our methods, but we coped with that by assuming that, somehow, the ocean floor was simpler than the land, the gradients of change more gentle, the processes not so variable in time and space. We thought subconsciously of the sea floor as having the gentle and smooth slopes of our sounding records and were startled by the discovery of vertical fault scarps and of open fissures. Similarly, the petrologists, having attributed to variations in the earth's mantle the subtle differences between basalts from various parts of the mid-ocean ridge, were surprised to find this entire range exhibited in a single 3 km long traverse across the volcanic axis, presumably as a result of ageing of the magma chamber. In a limited way, but we have no other means, we can

test the value of the old data by inquiring after their impact on our present understanding of the geology of the mid-ocean rift. I submit that this impact is negligible and no reason for comfort.

It does not seem proper to retreat one step and say "Well, the mid-ocean ridge is an active and hence complicated place but, surely, elsewhere in the ocean things are much more uniform and do not need this sort of detail". This, then, suggests that we should ask whether the detailed, fine-scale, near-bottom approach is the way of the future, rendering obsolete most of the old ways that bemuse our colleagues on land but are second nature to us. Aware that I am putting it very strongly I propose that the answer is yes and that we should, the sooner the better, divert most of our resources to this approach and leave the large scale global mapping to organizations charged with mapping. I am willing to allow for numerous exceptions but it seems that we have gathered enough regional data now and must proceed with the methods that provide the best hope to answer fundamental questions of process and history. I do not truly believe that we shall soon see the last of the roaming marine geologist disappear but I think that we should attempt to hasten his demise in order to look at the ocean floor in the detailed, time-consuming, expensive and, alas, often tedious way that will give us the answers we need.

Did retroviruses evolve from transposable elements?

from Andrew Flavell

RETROVIRUSES (formerly called RNA tumour viruses) have attracted much interest as they can cause cancer and are ubiquitous in a wide variety of vertebrates. It has long been known that DNA copies of these RNA-containing viruses are synthesized in infected cells and these copies are able to integrate into the cellular DNA, becoming part of the genetic complement of the cell. Recently the structures of the integrated 'provirus' DNA copies of several retroviruses have been studied by a combination of DNA cloning and sequencing methods (Shimotohno *et al.* *Nature* **285**, 550, 1980; Dhar *et al.* *Proc. natn. Acad. Sci. U.S.A.* **77**, 3937; 1980; Sutcliffe *et al.* *Proc. natn. Acad. Sci. U.S.A.* **77**, 3302; 1980; Majors *et al.* *Cold Spring Harb. Symp. quant. Biol.* **45**, 1980; Benz *et al.* *Nature* **288**, 665; 1980; Czernilofsky *et al.* *Nucleic Acids Res.* **8**, 2967; 1980). These results have shown that there exists a striking similarity between these structures and those of 'transposable genetic elements' from both

prokaryotes and eukaryotes. Transposable elements themselves are a highly interesting class of sequences (see *Nature* **287**, 188; 1980). In bacteria these sequences are able to move around inside the host cell genome and can catalyse chromosomal deletions and rearrangements. In *Drosophila* they can probably act as insertional mutagens (Gehring and Paro *Cell* **19**, 897; 1980; Rubin and Bingham, personal communication). Furthermore, the generation of antibody diversity in mammals, the control of mating type in yeast and alteration in surface antigens in trypanosomes all occur via DNA rearrangements which may share features in common with those of transposable elements. Lastly, tumour progression is often accompanied by chromosomal rearrangements, though no evidence exists connecting such changes with transposable elements; indeed their presence has not yet been proven in vertebrates.

All eukaryotic transposable elements and retrovirus proviruses studied so far possess DNA sequence repeats a few hundred nucleotide pairs long at each end of the element (see the figure). This

The generalized structure of a eukaryotic transposable element. The repeat units (>) are in the same orientation as the DNA and each repeat is itself bounded by small inverted repeats (I) of between 2 and 12 nucleotide pairs.

structure is also shared by some bacterial transposons although there seems to be a much broader spectrum of transposable structures in bacteria. It therefore seems tempting to postulate a familial relationship between all these various species. This idea has been leant further weight by DNA sequencing of several eukaryotic transposable elements and integrated retrovirus proviruses at the presumed key regions for transposition, namely the ends of the elements. Here, the (so far) inviolable rule is the presence of the dinucleotides TG CA at the ends of the element. This homology is much greater in several cases, notably that of the *Drosophila* element *copia* which shares with an avian retrovirus the sequence TGT TACAAACA at its ends (Levis *et al.* *Cell* **21**, 581, 1980; Shimotohno *et al.* *Nature* **285**, 550, 1980). Such similarities are very unlikely to be coincidental but cannot be regarded as proof of an interrelationship between these far-diverged species. Not surprisingly, some effort is being expended to relate these two fields by discovering whether eukaryotic transposable elements are really a special form of integrated retrovirus provirus or whether retroviruses are, in fact, transposable elements with an extra trick up their sleeves. The article in this week's *Nature* by Keshet and Shaul (see page 83) describes a family of DNA sequences in mice that may be both types of element simultaneously. Originally described by Howk *et al.* (*J. Virol* **25**, 115; 1978) these sequences (termed VL30 genes) were assumed to be retrovirus-related (although no sequence homology was detected with any retrovirus) by the ability of their RNA transcripts to be packaged into helper retrovirus virions which could be used to infect other cell types. Keshet and Shaul have now shown that those sequences possess direct DNA repeats at their ends. What is the relationship of this sequence family to the proviruses and transposable elements?

In 1970 Temin discussed in his proviral hypothesis the possibility that retroviruses might have evolved from an ancestral gene by successive cycles of transcription, reverse transcription and reintegration (*Persp. Biol. Med.* **14**, 11; 1970), each cycle picking up extra genetic information. In this light, such sequences as the VL30 family and perhaps some defective retroviruses might be regarded as intermediates in this pathway. It also seems plausible that retrovirus evolution might have proceeded via DNA transposition,

Andrew Flavell is in the Imperial Cancer Research Fund Laboratories, Mill Hill, London.

each new transpositional event adding to the repertoire of the element until its RNA transcript possessed the capsid and reverse transcriptase genes necessary for a successful sortie outside its home cell. Or possibly a combination of the two processes might be used. However, the answer to such crucial questions as 'Do RNA copies of the yeast and *Drosophila* elements ever leave their cells?', and 'Can retroviral proviruses move around their host genome without recourse to an RNA intermediate?' are as yet unknown. What is

not in doubt, however, is that both types of species, by their ubiquity, sheer quantity (it has been estimated that 5 per cent of the *Drosophila* genome is composed of *copia* -like sequences) and intriguing properties are well worthy of further study. To prove that retroviruses and transposable elements can interconvert in either direction it will be important (and difficult!) to find examples of the 'missing links' between them, one of which may well be the V130 gene family described in this issue. □

In the beginning

from E.G. Nisbet and C.T. Pillinger

OUR exploration of Darwin's "warm little pond" in search of the origins of life on Earth has revealed but a few islands of fact. New and apparently firm ground slowly appears: sometimes it turns to quicksand. In this issue of *Nature*, Bridgwater *et al.* (see page 51) cast doubt upon earlier reports of evidence for life in 3,800-Myr old rocks from Isua, Greenland. Here we examine some of the new evidence concerning the existence of life in the early Precambrian, and discuss recent advances in the understanding of the nature of the early surface of the Earth.

When did life begin? In an earlier review, Nisbet (*Nature* **284**, 395; 1980) concluded that the oldest possible evidence of life was from Isua, the oldest probable evidence from 3,500-Myr old rocks in W. Australia and the oldest definite evidence from 2,700-Myr old strata. Much work has been done on the Isua rocks and three lines of evidence have emerged: Pflug and Jaeschke-Boyer (*Nature* **280**, 483; 1979) have described apparent 'microfossils'; hydrocarbons have been reported among chemical extracts (*Chemical Eng. News* **75**, 22; 1979); and isotopic studies on carbonates (Schidlowski *et al. Geochim. Cosmochim. Acta* **43**, 189; 1979) suggest biological controls on the carbon cycle.

Pflug and Jaeschke-Boyer detected cell-like inclusions in cherty layers from Isua. They investigated these by the innovative use of the Raman laser molecular microprobe, an excellent approach unifying the efforts of palaeontologists and organic geochemists. The microprobe showed spectral features indicative of aliphatic side chains and esters. From this and their petrographic study Pflug and Jaeschke-Boyer concluded that the inclusions were microfossils of a primitive organism which they termed *Isuasphaera*. If so, this would be the oldest evidence of life.

Bridgwater *et al.* have examined duplicate samples of the same rocks. From

morphological and petrographic studies and the known metamorphic history of the rocks, they conclude that *Isuasphaera* are no more than fluid inclusions of secondary origin.

Much of the debate hinges on the metamorphism; Isua rocks may have reached temperatures as high as 500-600°C. Perhaps more important, oxygen isotopic data (Perry *et al. J. Geol.* **86**, 223; 1978) suggest minimum alteration temperatures of 390°C. Bridgwater *et al.* point out that high grade metamorphism does not entirely preclude the survival of microfossils. However, individual geolipids and esters could not be expected to remain intact after experiencing such conditions. In another paper querying Isua studies, Nagy *et al.* (this issue of *Nature* page 53) indicate the life expectancy of a straight chain aliphatic hydrocarbon at 600°C as about 10 seconds! In the oil industry it is well known that by 250°C everything that can be cracked will be cracked to give methane and graphite end products.

What then is the origin of Isua organics? Ponnampuruma (personal communication to C.T. Pillinger) has argued that hydrocarbons present in extracts of the acid resistant residues of Isua rocks must be contemporary because repeated and prolonged extraction of the intact rock matrix failed to release them (see also Barlow *Open Earth* **9**, 16; 1980 for a similar report). On the other hand Nagy *et al.* suggest that the extractables are no more than a few tens of thousands of years old and derive from encrusting lichens and cyanobacteria.

The lessons of contamination have been painfully learned by organic geochemists. Metamorphism may not be a problem of similar magnitude but it is noteworthy that Hayes *et al.* (Hayes, Kaplan and Wedeking, in preparation), from a painstaking study of H/C ratios in over 400 Precambrian samples, have concluded that all early Archaean samples investigated by them have been affected by thermal events. The H/C data suggest that

if evidence of biological activity survives in early Archaean rocks, it must be within the kerogen (the cross-linked organic polymer) rather than extractable geolipids. Stable isotope studies by the same investigators on 50 samples, selected as worthy of kerogen isolation, should produce interesting results.

Thus recent work has thrown much doubt on the supposed evidence for life in Isua time. Nevertheless, there is carbonaceous matter, albeit graphite, in Isua rocks, and the third category of evidence, isotopic studies on the carbonates, remains intriguing. Schidlowski *et al.*'s work is not inconsistent with biological processes in a stabilised carbon cycle. The evidence remains 'possible'.

In slightly younger rocks, the information is becoming much more certain. There are three well-studied 3,500-Myr old terrains of low metamorphic grade — the Pilbara Block, Australia; the Barberton Mountain Land, South Africa; and parts of the Rhodesian Craton, Zimbabwe. In all three, a variety of sedimentary rocks exist, some of which are remarkably little deformed and metamorphosed, in contrast to the samples from Isua. In the Pilbara, new mapping, both by Hickman and Lipple (Geological Survey of W. Australia), and in the very important field program of the University of W. Australia, has led to the discovery of much evidence of 3,500-Myr life, especially in rocks collected by J. Dunlop and R. Buick (University of W. Australia). Dunlop *et al.* (*Nature* **274**, 676; 1978) found possible microfossils and Walter *et al.* (*Nature* **284**, 443; 1980) found a probable stromatolite structure. Significantly, important new discoveries have been made by Awramik, Walter, Schopf and co-workers in rocks from areas studied by Dunlop and Buick (*Geotimes* Sept, 1980). This work, "finding the first conceit of love there bred, where time and outward form would show it dead", is becoming accepted as sound evidence for early life. It may be that as early as 3,500-Myr ago there was an abundant, simple but diverse biological community in the seas.

So much for the islands of 'fact'. What of the warm pond of speculation? Recent modelling (often by Monte-Carlo methods) of early Archaean conditions is helping to place constraints on that pond. Frey (*Precamb. Res.* **10**, 195; 1980) and Grieve (*Precamb. Res.* **10**, 217; 1980) have suggested, though with different models, that late heavy bombardment of the Earth by asteroid sized bodies established a topographic dichotomy between 'continents' and 'oceans' by about 4,000-Myr ago. Former models proposing a global ocean, such as that of Hargraves (*Science* **193**, 363; 1976) appear unlikely; the process described in *Psalm* 104, 5-9 appears to have taken place very early indeed.

Models of the atmosphere have also

improved. In recent years there has been a strong shift away from the traditional idea that the early atmosphere of the Earth consisted of a highly reduced mixture of methane, ammonia and hydrogen. If a reducing atmosphere did once exist, it would probably not have survived for long, ammonia being very rapidly converted by photolytic destruction to nitrogen. Hart (*Icarus* 33, 23; 1978), Owen *et al.* (*Nature* 277, 640; 1979) and Henderson-Sellers and Schwartz (*Nature* 287, 526; 1980) have shown that the 'window of life' must have been rather narrow: if the atmosphere had had too much CO₂ or if temperatures had been slightly higher a Venus-like runaway greenhouse inferno would have developed; too little and Martian-like glaciation would have occurred. Perhaps only in a relatively

short period around 4,000-Myr ago were surface conditions suitable for life to appear: once it had appeared, the CO₂ levels would be controlled and the danger of runaway averted. Henderson-Sellers and Schwartz also highlighted the ammonia 'problem' — there was probably very little in the atmosphere, except perhaps small quantities produced abiotically by photochemical reduction of N₂ catalysed by TiO₂ in desert sands. Consequently, those chemical evolutionaries who rely on the availability of ammonia may depend on the uncertain intervention of a *deus ex titania*. Life did appear, the warm pond was occupied, but it may have been a close-run thing. Perhaps the Earth is indeed a very rare place, perhaps there are few Gardens in Eden. □

Clin-Midy, Montpellier) call them. The toxins, exemplified by diphtheria toxin, abrin and ricin, consist of a B piece which has a receptor for the cell surface and which may mediate the transport into the cytoplasm of the A piece (Olsnes *et al.* *Nature* 249, 627; 1974). The A pieces are inhibitors of protein synthesis which can in various ways interfere with messenger RNA translation in cell free systems and which are presumed to do the same in the cytoplasm of cells to which they gain entry (Pappenheimer *Ann. Rev. Biochem.* 46, 69; 1977).

The means of linking together the toxin and carrier was discussed. Covalent binding of some sort seemed indicated and various chemical strategies for achieving this were considered. Gros (Clin-Midy) reported that a disulphide bond, such as exists naturally between A and B chains of toxins, was needed to link together ricin A chain and immunoglobulin in a form that could express cytotoxicity in tissue culture. The stability of the SS link *in vivo* was questioned and dissociation of the conjugate may explain the lack of impressive therapeutic results with A chain conjugates in animals. Edwards (Wellcome Foundation, London) reported that use of a disulphide bond to attach intact abrin to anti-lymphocyte globulin yielded a conjugate less capable of inducing immunosuppression in mice than a chlorambucil-linked conjugate.

The membranologists, particularly Blobel (Rockefeller University), were of the opinion that the only molecules that would be able to cross the membrane barrier would be those which had the appropriate translocation signals. The B pieces of toxin molecules have been widely regarded as possessing the signal for translocation of the A chain from outside the cell into the cytosol. This may also be true of diphtheria toxin since conjugates with the isolated A chain have lacked potency (see, for example, Gilliland *et al.* *Proc. natn. Acad. Sci. U.S.A.* 75, 5319; 1978; Chang *et al.* *J. Biol. Chem.* 252, 1515; 1977). But it seems from the work of Jansen (Jansen *et al. Immunol. Lett.* in the press) and Thorpe (Institute of Cancer Research, London) and others (Uchida *et al.* *J. Biol. Chem.* 255, 6687; 1980; Yamaguchi *et al. J. Nat. Cancer Inst.* 62, 1387; 1979) that conjugates with ricin A-chain or A-piece like materials such as gelonin (Stirpe *et al. J. Biol. Chem.* 255, 6947; 1980) can also be profoundly and specifically cytotoxic. Antibody molecules, like many if not all other secreted proteins, are thought to lose their leader sequences at the time of their translocation into the vesicle from which they will be shed. Thus it must be considered that certain A pieces, with antibody-conferred capacity to bind to cells, may themselves have the additional information necessary to be translocated

Magic bullets

from Tony Davies

THE possibility of constructing Zauberkugeln — magic bullets — was first thought of by Paul Ehrlich in his inspired attempts to find new cures for a variety of human diseases. Magic bullets, he said, would have some of the properties of the antibodies then newly discovered by Behring and would be able to find and destroy a target in the body which was responsible for a particular disease.

At a recent meeting* (part of the *Quo Vadis?* series) progress towards the fulfilment of Ehrlich's dream was considered. The aim of the organisers was to assemble a group of membranologists and cancer chemotherapists, immunologists, experimentally orientated clinicians and toxinologists who might collectively constitute an engineering team to consider the numerous component problems of the overall design.

The basic philosophy is to have a carrier molecule, of which the function is selectively to bind to the target, covalently attached to a 'warhead' chosen to kill or modify the target cells. Carrier molecules could be antibodies, lectins, peptide hormones or any other molecule which tissues take up selectively but consideration was limited to the use of antibodies and, in particular, monoclonal antibodies.

There was agreement that the homogeneity afforded by monoclonality was advantageous but restriction of the number of sites to which such a highly specific antibody could bind was seen as a potential problem. Localisation is, however, fundamental to success and it was demonstrated by Mach that localisation of anti-CEA antibodies onto human colonic carcinomas in either mice or men could sometimes be demonstrated (Mach *et al. New Eng. J. Med.* 303, 5; 1980). In well differentiated tumours it

tended not to occur, presumably because of difficulties of access to the luminal (antigenic) surface of the malignant ductal epithelium. It is not known whether the larger molecular weight conjugates would do any better but common sense seems to indicate that they would not. The obstacle is not, however, insuperable in well delineated tumours as various devices could be employed to improve local blood flow or to promote extravasation. Neither is it impossible that IgE anti-tumour antibodies could be used as systemic promoters of extravasation. Alternatively IgA antibodies might be transported across the epithelial cells to the target surface as long as the mechanism for secretory piece recognition could operate.

Warheads so far utilised include small molecular weight standard chemotherapeutic drugs or high molecular weight toxins or their subunits. Enzymes have also been employed.

Toxic agents of small molecular weight have limited effects because relatively few molecules can be bound to the carriers without reducing their cell binding capacity. This problem might be circumvented by binding relatively large numbers of drug molecules to a macromolecule such as poly L-glutamate (Rowland *et al. Nature* 255, 487; 1975) or dextran (Rowland *Eur. J. Cancer* 13, 593; 1977; Hurwitz *et al. Int. J. Cancer* 21, 747; 1978) and then attaching the complex to the carrier with target specificity. It emerged incidentally that merely binding daunomycin to dextran reduced the toxicity of the drug although without apparent loss of therapeutic potential. The mechanism involved here probably involves a slower release of the drug over a long period of time rather than an alteration in its pattern of distribution.

Excitement at the meeting was caused by the description of antibody-toxin conjugates or 'immunotoxins' as Jansen and his colleagues (Centre de Recherche

*The meeting held in Montpellier, 17th July, 1980, was a satellite symposium of the International Congress of Immunology in Paris.

Tony Davies is Professor of Immunobiology, Institute of Cancer Research, London.

into the cytoplasm without a mediating B-piece.

The technology of antibody-toxin conjugates is developing at a time when our understanding of plasma membranes is increasing rapidly. At present we simply do not know exactly how or why toxins gain entry to cells although we can make informed guesses. The consequences of binding of antibody to cell surfaces are also only known in a limited number of cir-

cumstances, so it is very difficult to predict what will happen to an antibody molecule coupled to a toxin.

The overall impression was that the new breed of conjugate therapists should increase their efforts to attack targets *in vivo*, in experimental models, perhaps taking into account the pattern of potential clinical usage. The existing results are promising and the answer to the question 'Quo Vadis?' seems to be 'forward'. □

Macroevolution

from John Maynard Smith

IN October 1980 a conference was held in Chicago on "macroevolution". At the meeting, some 150 palaeontologists, anatomists, taxonomists, embryologists and geneticists debated the claim that the large-scale features of evolution cannot be explained as a mere extension of the micro-evolutionary changes within populations which are studied by geneticists.

The main proponents of this view have been Niles Eldredge, Steven Gould and Steven Stanley. The minimum claim is an observational one, that the fossil record does not show a gradual transformation of populations, but instead shows 'stasis' — morphological constancy of species over millions of years — and 'punctuation' — the sudden appearance of new species.

To establish this assertion would require measurements of the distinguishing characteristics in populations from successive strata. An odd feature of the meeting was that no data of this kind were presented. Stanley's book, *Macroevolution*, argues for punctuation mainly on the basis of data on the durations of taxonomic groups (species, genera etc.) in the fossil record. The trouble with this is that one does not know whether one is studying the habits of taxonomists or of evolving taxa. The morphometric data he presents (notably, Gingerich & Simons' data² on adapid mammals) show gradual transformation rather than stasis and punctuation. The claim, therefore, appears to rest on the strong impression felt by some, but by no means all, palaeontologists; it may be true, but needs to be shown to be true.

One study known to me which does support the punctuationist view is that of Williamson³ on 21 species of fresh-water molluscs in the lake Turkana region of Africa. Over the last 5 million years population measurements show only small changes in 16 of the species. The other five change little for most of the time, but

rapidly during a period of perhaps 50,000 years when the lake was isolated. The derived species later went extinct and were replaced by the original forms, presumably by immigration from outside. This example illustrates a semantic difficulty which often confused the conference. A new species arising in 50,000 years (2m of sediment) is sudden to a palaeontologist but gradual to a geneticist. As a geneticist I was left with the impression that the 'sudden' appearance of new species called for no new explanations, but that stasis may do.

Punctuations have made a further deduction from the supposed pattern of stasis and sudden change. This is that long-term trends in evolution are the consequence of 'species selection'. According to the hypothesis of species selection, the species is the 'unit of selection', which either reproduces (that is splits into two species) or dies (that is goes extinct); this is in contrast to the more usual view, according to which the individual is the unit of selection. It is proposed that some lineages show greater speciation rates and/or lower extinction rates than others, and that therefore there is a trend towards those characteristics responsible for rapid speciation and low extinction. Some of the most interesting data presented concerned associations between species' traits (for example, sedentary versus planktonic larvae) and rates of speciation.

The deduction that species selection is the sole cause of trends rests, however, on the further assumption that punctational changes are as likely to occur in a direction opposite to that of the trend as parallel to it. If this assumption is not true, then the overall trend of evolution would be the sum of the punctational changes, each one of which would be the result of selection at the individual level within the populations. The punctational patterns of change would then not imply any new evolutionary mechanism. It would merely show that evolution occurs not at a uniform but at a very varied rate. This would hardly be new; it was in fact the main theme stressed by

G.G. Simpson, one of the founders of the 'modern synthesis'.

There is no evidence to show that the direction of punctational changes is random with respect to major trends, and it seems implausible as a generalisation. It implies, for example, that in the evolution of mammals from reptiles, newly derived species, when compared to their ancestors, were as likely to have more outwardly directed femur and humerus than the opposite, and similarly a shorter secondary palate, a more complete dermal covering to the skull, more bones in the lower jaw, and so on for all the other trends.

Stasis, punctuation and species selection constitute the minimum claim of the new school. Further claims are that the reason for stasis lies in 'developmental constraints', that the origin of new species requires 'hopeful monsters' (large morphological changes arising from a simple genetic change) and/or 'genetic revolutions' (major reconstructions of the genome, perhaps occurring in a small population), and that the fundamental structures, or bauplans, of major taxa represent a set of possibilities constrained by the laws of development; in contrast, Darwin argued that the structures common to a taxon exist because they were present in the ancestors of that taxon, and that they arose in those ancestors as naturally-selected adaptations to particular ways of life.

Thus there are two possible ways of explaining stasis; normalising selection (that is, selection favouring the typical members of a population relative to the extremes) or developmental constraints. Species did not change either because deviants were weeded out, or because the developmental system prevented them from arising. Clearly both effects operate. The crucial test of their relative importance lies in a study of geographical variation in existing organisms. Thus it seems to be the case that species with a wide geographical distribution often display a range of forms such that the most extreme differ from one another as much as do sympatric species. If so, it is hard to see how developmental constraints can account for stasis. There is a clear need to compare patterns of spatial and temporal variation in the same groups.

Is there evidence that macromutations have been important in speciation? The best way to find out seems to be a genetic analysis of hybrids between morphologically distinct species. One example quoted in Chicago concerns hybrids between *Drosophila sylvestris* and *Drosophila heteroneura*^{4,5}; the latter has projecting eyes which are among the most striking morphological variants in the genus. Analysis shows that the difference

John Maynard Smith is Professor of Biology at the University of Sussex.

1. Stanley, S.M. *Macroevolution: Pattern and Process*. (W.H. Freeman, 1977).
2. Gingerich, P.D. & Simons, E.L. *Univ. Michigan Mus. Palaeont. Contrib.* 24, 245 (1977).
3. Williamson, P. thesis, Univ. Bristol (1980).
4. Val, F.C. *Evolution* 31, 611 (1977).
5. Templeton, A.R. *Evolution* 31, 630 (1977).

in eye shape is caused by genes at at least eight loci. It seems that no macromutation was involved in this case. I know of no example pointing the other way, but shall not be surprised if one crops up.

Genetic analysis cannot, of course, be applied to the more extreme differences between bauplans, so it is still possible to argue that the origins of major taxa require special genetic events. However, no-one at the conference was prepared to challenge Stebbins's remark that the most striking morphological novelty among the higher plants was the flower head of *Zea mays*, which is largely the result of selective

breeding under domestication and is known to be under the control of many genes with small effects.

It will be apparent that this report has been written by a geneticist, not a palaeontologist. Despite my reservations, I found the meeting immensely stimulating. It can only be good for evolutionary biology that people from such different disciplines should meet, talk and, occasionally, listen. It was only to be expected that there would be much misunderstanding, confusion and even indignation. I hope we shall meet again in a year or two, and that next time the confusion may be slightly diminished. □

leaves the species somewhat vulnerable to winter frost; its distribution appears to correlate well with the isotherm of the main daily maximum temperature of the coldest winter month at 0°C, and also with a mean daily maximum of the warmest summer month at 25°C. Its sensitivity to winter frost and summer drought account for its western distribution and its confinement to the woodland habitat, where it is protected from both. The survival of bluebells in open habitats in the extreme west of Britain (often as a relict of former woodland) may be a reflection of the mild, oceanic conditions prevailing there.

The examination of seed from one further woodland species, the wood millet grass, *Millium effusum*, has shown a rather more complex pattern of reaction. Thompson (*Ann. Bot.* 46, 593; 1980) found that seed germinated immediately after harvesting had an optimum temperature requirement of 16°C but, after storage for 2.5 months, the optimum temperature for germination was 21°C and germination took place over a wider temperature range. The breadth of tolerance increased yet further after 10 months. Also, germination was faster after a period of storage but, rather surprisingly, a higher temperature (26°C) of storage reduced the subsequent germination rate. The ecological significance of this high temperature inhibition of the after ripening process is difficult to discern, but Thompson suggests that it could reduce the chances of an ill-advised, rapid response to mild conditions soon after shedding.

Millium effusum has a wide distribution in Europe and, in view of the late flowering and fruiting in the north of its range (late August in areas to the north of the Baltic), temperatures will already be below those needed for germination, which is thus delayed until spring. In the south (southern France and Iberia), seed is shed in early summer, so it experiences a hot, dry period followed by a warm, moist one. This results in the bulk of the seed germinating before winter arrives. In between these two extremes, the complex microclimatic variations caused by site factors (microtopography, aspect, etc.) and fluctuations in weather conditions, can result in a range of responses. Indeed, the plasticity of this species may be a key to its success.

Thompson's germination studies illustrate the possibilities which exist for pinpointing the limits of a plant's capacity to meet the stresses of the environment, but seed germination is only one of many critical phases in the plant's life cycle. Also, the fact that one factor may limit a species on a continental scale, does not preclude the possibility that other factors will limit its local distribution pattern. At the level of the individual one must still account for the part played by chance. At this scale is vegetation simply, in the words of Gleason, a "fortuitous juxtaposition of plant individuals"?

Why is a plant where it is?

from Peter D. Moore

ONE of the simplest questions to ask, yet one of the most difficult to answer is why a particular individual of a given plant species should be found growing at a particular location. The answer may be sought in its physiological requirements and the degree to which they are satisfied by the physical framework of that spot; or in the complex of biological interactions with other organisms, competitors, predators, parasites, and so on; or in the tangled web of historical events which has moulded both of these former factors; or, finally, in the sheer chance which brought a propagule to that site and which left it untouched by random catastrophe. The interaction of all these forces permits no easy answer to the apparently simple question.

Any attempt must seek first to locate the physical environmental stress and physiological sensitivity which, in combination, allow the identification of a limiting factor. This factor can be regarded as a necessary resource in such short supply that any increase in its availability will result in an increase in the performance of the individual or density of the population. But the model, stated in these terms, needs to be flexible enough for such factors as temperature at specific times of day or year to be regarded as a resource.

One may examine any aspect of the plant's physiology, or any time in the plant's life history, to find where resource restriction meets the plant at its most vulnerable point and prevents further reproduction, growth, or even survival. Perhaps the most obvious place to start is at the beginning and to define those resource limitations which restrict the germination of the seed and thus determine whether the plant is to be given the opportunity to meet further tests. Thompson (*Ann. Bot.* 34,

427; 1970) has approached from this direction in his studies of various species of Caryophyllaceae. He devised a metal bar along which a temperature gradient was maintained and observed the germination characteristics of each species in relation to temperature, and found that their requirements correlated well with the biogeographical characteristics of each species. For example, a species (*Silene secundiflora*) from the Mediterranean germinated at low temperature, this being advantageous to species in which autumn germination means a mild, damp period before meeting the unfavourable drought of the following summer. A more northerly species, on the other hand (*Lychnis flos-cuculi*), had a higher temperature germination requirement; it thus remains dormant through the winter after seed set and germinates in the spring.

Subsequently Thompson (*Ann. Bot.* 39, 1; 1975) has examined in detail the geographical variation between populations of a single species, namely *Silene dioica*. He has found considerable variation both within and between populations and also a different response among after ripened seeds from those which were fresh. The populations from regions with milder winter contained more individuals which were capable of germination at low temperature, thus confirming the trend indicated by the previous experiments.

Silene dioica, the red campion, is a woodland herb, widespread in Europe, which, in the western, deciduous woods of Britain and northern France is often accompanied by the bluebell, *Hycanin-thoides non-scriptus*. The seeds of this species were also subjected to the attentions of Thomson and Cox (*Ann. Bot.* 42, 51; 1978), and on the basis of their experiments they propose that the seeds germinate naturally at about 11°C, in the late autumn and that they therefore overwinter as seedlings. This obviously

Peter D. Moore is in the Department of Plant Sciences, University of London King's College.

Galileo would have been pleased

from J. E. Guest

THE discovery that Io, the Moon-sized satellite of Jupiter, has active volcanoes throwing plumes as much as 270 km above the satellite's surface was one of the exciting revelations made by the Voyagers I and II spacecraft as they sped past Jupiter and its attendant satellites in March and July 1979. The four largest satellites in the Jupiter system, named Io, Europa, Ganymede and Callisto, were discovered by Galileo in 1610. For three centuries after their discovery little more was found out about them but, during this century, it became abundantly clear, even from Earth-based astronomical observations, that each satellite has distinctive characteristics.

Although classified as satellites, the Galilean moons are planetary bodies in their own right and of similar size to the Moon and Mercury. Io, the innermost of the satellites, was thought to be the most peculiar. Its density ($3,500 \text{ kg m}^{-3}$) is similar to that of terrestrial rocks and its surface is characterised by high reflectivity and a striking reddish colour. Furthermore, Io is constantly emitting atoms from its surface, leaving a trail of ionised sulphur, potassium, hydrogen and sodium behind it to form a torus-shaped atmosphere in its orbital path. Telescopic observations of the next satellite outwards, Europa, showed that it too has a density ($3,100 \text{ kg m}^{-3}$) consistent with it being made primarily of rocks. Its density is slightly less than that of Io implying a higher water content. Whereas Io is somewhat larger than the Moon, Europa is almost the same size.

The way light is reflected from Europa and the next satellite out, Ganymede, suggests a covering of water ice on their surfaces. It is thought that Europa is between 50 and 100 percent frost and ice, and Ganymede between 20 and 60 percent covered by fresh ice. Ganymede has a much lower density than Io or Europa, ($1,900 \text{ kg m}^{-3}$) implying a high content of water and ice. Callisto, on the other hand, which is of comparable density ($1,800 \text{ kg m}^{-3}$ from new Voyager data) to Ganymede, has a low albedo and any ice at the surface must be 'dirty' containing fragmental rocky material.

Thus from the limited pre-Voyager astronomical data it was clear that the internal structures and compositions, and the processes that had moulded the surfaces could be very different from those with which we have become familiar on the terrestrial planets. From previous studies of the Moon, Mars and Mercury, a familiar pattern of surface history for solid planetary bodies has emerged. The early surface history was dominated by impact craters, ranging from the minute to large basins of 13,000 km diameter or more. The surface expressions of any other processes

operating at the same time, such as volcanism, were destroyed. For the Moon at least, this period of bombardment ended around 4×10^9 years ago. There then followed for each of these planetary bodies a period when volcanism dominated to give on the Moon, for example, the extensive dark marial plains, consisting of vast sheets of lava. On Mars, the volcanic process was more varied giving large volcanic constructs such as the huge mountain of Olympus Mons. Tectonic processes produced patterns of faults, the most complex structures being on Mars. As well as these internal processes modifying the surface Mars was further modified by the effects of water and wind erosion as well as the effects of sub-surface ice. But even these processes occurred relatively early, the major events having been completed $2-3 \times 10^9$ years ago.

The pictures returned by the Voyager spacecraft show surfaces much stranger than had been predicted.

Whereas for bodies in the inner Solar System virtually the whole record of surface history has been preserved, the surfaces of the Galilean Satellites have each preserved different periods in their history and each is dominated by specific processes.

The outermost satellite, Callisto, has a surface dominated by impact craters and basins, and presumably has the oldest preserved surface of the Galilean satellites. The high content of ice and water suggests that there was little modification of the surface by internal activity following the period of impact bombardment. Ganymede on the other hand has a higher rocky content and as well as impact craters a complex pattern of tectonic features which have strongly modified the surface since the period of heavy bombardment can be seen. The evidence suggests that during the period of bombardment the mechanical crust of Ganymede was probably only a few tens of kilometres thick and there then followed a period of rapid thickening and stiffening of the crust resulting in the tectonic patterns now observed.

When we examine the surfaces of Europa and Io, we can see they have quite different histories. On Europa only a few impact craters have been identified suggesting that the present surface was formed after the period of intense bombardment; on Io, volcanic resurfacing has obliterated the craters formed earlier. Thus, although on Ganymede internal activity has been important, its effects on the sur-

face were concluded early in its history, whereas for Europa and Io, internal mechanisms have continued, and, in the case of Io are still operating.

Models for the internal constitution and thermal history of the two inner Galilean satellites have concentrated on two energy sources, one deriving from tidal interaction between Jupiter and the satellites and the other involving decay of long lived isotopes within the rocky interiors. Models involving these two processes depend very much on the satellites' origin, the timing of events and the composition and degree of differentiation.

In their remarkably well timed paper, Peel *et al.* (*Science* 203, 892; 1979) produced a model in which there was intense heating in the interior of Io by tidal dissipation, anticipating the Voyager results of active volcanism on Io just a few days before it was discovered. Likewise, tidal heating can have played a part in the thermal history of Europa and tidal deformation may explain some of the surface characteristics. The surface of Europa is generally smooth and bright with a strange pattern of intersecting linear features composed of shallow grooves, low ridges and albedo markings. The topographic relief on these features is small and only seen close to the terminator. These features are probably formed by stress in the surface materials, but their orientation has not so far been shown to be uniquely explicable in terms of a particular stress field. Previous models for the internal constitution of Europa suggested that early differentiation resulted in a silicate core surrounded by a liquid water mantle, crusted over by some tens of kilometres of water-ice. However, more recent calculations suggest that solid state convection in the crust probably caused progressive freezing of the liquid mantle and it now seems unlikely that tidal heating could have maintained the liquid state.

The Voyager discoveries have prompted a reconsideration of this type of model. It is possible that the six percent by weight water component (derived from density considerations) is not all taken up as free water or ice at the surface, is instead contained in hydrous silicates in the rocky part of the planet, leaving only a thin ice outer shell of a few kilometres thickness (Ransford, *et al.* this issue of *Nature* page 21). However, such a model imposes constraints on the time of formation of the surface ice. It appears that Europa's surface consists of relatively 'clean' ice, although in some areas a brownish hue to the surface may suggest contamination by rocky material. Assuming that Europa, like the outer Galilean satellites, was bombarded in its early history it is

J.E. Guest is a lecturer in Planetary Science in the Physics and Astronomy Department, University College London.

inevitable that, with a thin icy crust underlain by rock, impact excavation would have brought up rocky material, mixing it with the surface ice: the present ice layer must therefore have formed after the period of heavy bombardment. With a thicker ice (or water and ice) outer shell, impact need not have penetrated to the rocky layers and the effects of impact might have been removed by cold viscous flow in the ice crust. Alternatively, early formed craters might have been eroded by the sputtering effect of charged particles capable of removing tens of metres, or even kilometres, from the surface (Smith *et al. Science* **206**, 927; 1979). A thin icy crust must either have formed after the end of bombardment, or have remained in a liquid state, allowing denser silicate

material to settle, until after the bombardment period.

The origin of the linear pattern on Europa is particularly enigmatic. Various proposals have been put forward including crustal expansion owing to freezing of an early ocean, global expansion as a result of dehydration of the interior, and tidal deformation. The complexity of the pattern made up of features of different morphology is difficult to interpret in terms of mechanism and indeed more than one process may have contributed to the surface features. At present, interpretation of the surface of Europa is model-dependent; a situation which is perhaps not surprising on a planet whose geology is unlike anything in our previous experience. □

were not contiguous cyanobacteria strains. It is unclear from the published data for *nif* gene hybridization whether or not all three structural *nif* genes (H D and K) hybridize in all these species. Hopefully this will be resolved in the near future.

Another problem which should be resolved soon is the determination of the extent of DNA sequence homology for these genes in different species. The DNA sequences for the *nif* H proteins of *Anabaena*, (Haselkorn pers. comm.) and *Klebsiella* (Ausubel pers. comm.) are now available. It will be interesting to see how highly conserved the sequences of the different genes are and whether or not the data support the idea that the enzyme has been conserved over a long evolutionary period, or that nitrogen fixation is a relatively new function which has been transferred between different bacterial genera.

These DNA hybridization studies have been of very great value for comparative studies of *nif* genes in different families. As described above, they can be used to determine whether *nif* genes are chromosomal or plasmid borne, whether they are linked to each other and as a method for isolating *nif* DNA for studies of its expression in different hosts or for further genetic studies. It is these last two uses of the *nif* hybridization work which are discussed by Ruvken and Ausubel in this issue of *Nature*. They show, as one would expect, that the *K. pneumoniae nif* DNA can be used to isolate the corresponding *Rhizobium* genes and introduce them into *Escherichia coli*. Of much greater importance scientifically is the demonstration that this procedure can be used to induce mutations in the *Rhizobium nif* DNA within *E. coli* and then return the defective gene to its original host where recombination can be forced so that the defective gene is introduced into the chromosome replacing the existing functional gene. In this way mutants of *Rhizobium*, or potentially any other nitrogen fixing prokaryote, which are defective in known *nif* genes can be induced.

Once such a mutation has been induced it is possible to re-isolate DNA fragments extending from this region and induce mutations in them. By knowing the extent of the overlap with the original fragment a series of mutations can be induced at known distances from the original mutation by repeated rounds of mutagenesis and replacement of the corresponding host DNA. While the value of this technique for studies of genes linked to a particular *nif* gene is obvious it is also equally valuable for all species where cloned DNA fragments are available and there is a method for re-introducing the mutated DNA into the host. Thus we now have a technique which could be used to prepare maps of chromosomes or plasmids in a wide range of species of microorganisms. □

Finding nitrogen fixation genes

from John E. Beringer

THE enzymic conversion of atmospheric nitrogen to ammonia is carried out only by prokaryotic microorganisms. The structure and properties of the enzyme complex used, nitrogenase, are very similar in all species that have been studied.

Nitrogenase consists of two main components. One is an iron protein, composed of two identical subunits; it binds MgATP and transfers electrons to the other component which reduces the substrate. The second component is a molybdenum-iron enzyme containing four proteins which can be separated into two distinct sub-units, giving the enzyme an $\alpha_2\beta_2$ type of structure. All these proteins are irreversibly denatured by oxygen. A further striking similarity between nitrogenases is that a functional enzyme can be constructed by mixing iron and iron-molybdenum proteins from different genera; for example *Klebsiella* and *Azotobacter*, though some combinations such as *Klebsiella* and *Clostridium* are inactive.

Over the last few years there have been dramatic advances in our understanding of the genetics of nitrogen fixation (Nif). This has been due largely to the fortunate finding that *Klebsiella pneumoniae nif* genes are linked to each other and could be manipulated in *Escherichia coli*. As a result of extensive mutant and complementation studies we know that there are 17 *nif* genes in seven or eight operons. Three genes code for nitrogenase proteins; *nif* H for the iron protein and K and D for the different subunits of the molybdenum-iron enzyme. Functions have yet to be assigned to some of the other genes.

Parallel genetic studies of *nif* genes in other nitrogen fixing species have been restricted by limitations in our ability to do

the necessary genetic manipulations or, in the case of the genetically amenable *Rhizobium* species, our inability to obtain nitrogen fixation in pure culture. However, using *Rhizobium meliloti* as an example, Ruvkun and Ausubel in this issue of *Nature* (see page 85) have shown that one can take advantage of the conservation in DNA sequence of *nif* genes to promote site-directed mutagenesis and localized mapping in prokaryotes.

In 1979 Nuti *et al.* (*Nature* **282**, 533) reported that DNA encoding the *K. pneumoniae* iron and molybdenum-iron proteins hybridized with plasmid DNA from *Rhizobium*, though DNA encoding most of the remaining *Klebsiella nif* genes did not hybridize. In January this year Ruvkun and Ausubel (*Proc. natn. Acad. Sci. U.S.A.* **77**, 191) and Mazur, Rice and Haselkorn (*Proc. natn. Acad. Sci. U.S.A.* **77**, 186) reported further evidence for the conservation of *nif* genes. Ruvkun and Ausubel looked at 19 strains of different nitrogen fixing prokaryotes, including Gram-positive and Gram-negative bacteria, cyanobacteria and the actinomycete *Frankia*. All showed DNA hybridization with the fragment of *K. pneumoniae nif* DNA carrying genes K D and H used by Nuti *et al.*, whereas no hybridization was observed with controls representing ten different non-nitrogen fixing species or with DNA carrying the other *nif* genes. Mazur, Rice and Haselkorn confirmed that hybridization could occur with three strains of cyanobacteria representing two different species. They further showed by heteroduplex studies of hybridized DNA that the genes

J.E. Beringer is in the Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, UK.

ARTICLES

Lineament and polygon patterns on Europa

David C. Pieri

Space Sciences Division, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91103

A classification scheme for the lineaments and associated polygonal patterns seen on the surface of Europa is presented, and the polygon frequency distribution discussed.

THE surface of Europa is dominated by lineaments intersecting to form polygons and by complex systems of low ridges which often exhibit cycloid planimetric patterns^{1,2}. Polygonal or nearly polygonal patterns are common in nature and are often associated with stress-relief fracturing. The interpretation that the albedo lineament patterns are associated with fracturing at different scales is used. Two major pattern groups are of interest: (1) roughly equant polygons formed by the surface expression of fractures formed in isotropic stress regions, both tensional and compressional (for example, shrinkage cracks in mud, cooling cracks in basalt, patterned ground, fracture patterns over salt domes); and (2) complex trapezoidal patterns formed by the intersection of faults or joints under directed stress.

The existence of either of these pattern types has implications for the state of stress under which each type formed. This article presents a classification scheme for the lineaments and associated polygonal patterns seen on Europa, and reports on the polygon frequency distributions. Possible fracture mechanisms and scenarios for the formation of lineament systems on Europa are examined in detail elsewhere³⁻⁵.

Lineaments

Figure 1 is a low resolution sketch map of the global lineament patterns on Europa. The general orientation of lineaments along great circles is apparent and has been mentioned previously (ref. 1 and Plescia personal communication). Lineaments are seen less frequently in low albedo regions, although this may be due to loss of contrast across dark areas.

Figure 2 is a high resolution (4 kilometre per line pair (km/lp)) mercator projection mosaic. Figure 2 shows that at small scale the lineaments form extremely complex patterns. Some lineaments exhibit relief while others seem to be primarily albedo features. Most dark lineaments possess a white medial stripe and often, when dark lineaments lie across low albedo terrain, only the white interior stripe is visible across the low albedo patch. The first step to try to understand these enigmatic features was devising a preliminary classification system which is outlined in Table 1 and Fig. 3. Although this classification is rudimentary it allows different styles of lineaments to be discussed. The lineaments have been divided on the basis of albedo, morphology, orientation, and characteristic geometric style into eight groups. However, this analysis is based entirely on the limited set of Voyager 2 images taken from a distance of ~100,000 km and the best resolution of the images is ~4 km. Stereo coverage, while available, is marginal and of low resolution. Coverage at 4 km resolution is available only for ~25% of the planet, the remainder of the surface is covered at resolution at least an order of magnitude worse. Although this limits the analysis the classification scheme is, nevertheless, representative of the region for which coverage is available.

Figure 3 shows the various lineament geometries and Table 1 gives type descriptions and locations. These lineaments on Europa are clearly of several length scales and there are at least two basic types. Flexi (Type 6) and scarps (Type 8) seem to be

topographic features with little or no albedo contrast to the surrounding terrain. The flexi are global features, and we do not know the extent of the low scarps. The linea appear to be primarily albedo features. Type 3 with white centre striations appear to have some positive relief although Soderblom (personal communication) reports that based on photometric measurements relief may be small to non-existent. Negative relief for one northern linea has been suggested². Lineament Type 3 is clearly global. These are generally large great circles. Type 1, 4, and 7 seem to be regional in extent, whereas Types 2 and 5 may be the result of highly localized processes.

Polygonal areas

All the lineaments mentioned above define a system of polygons varying in size from small (limit of resolution 10 km²) reticulate polygonal patterns to very large (10⁶ km²) individuals. The statistics of polygonal patterns in nature are well-known^{6,7}.

For macroscopic geological settings two pattern types are common in terrestrial experience: (1) equant five or six sided polygons; and (2) predominately four-sided polygons formed by faults or joints in complex settings.

The two types have distinct and characteristic frequency distributions for polygons of a given number of sides: the so-called Voronoi distribution (mode 6) is characteristic of polygonal patterns formed under uniform tension or compression; the Poisson distribution (mode 4) being characteristic of polygons formed by random intersections of lines on a plane or by the intersections of random great circles on a sphere^{6,8,9} (Fig. 4a).

Literature data on several types of terrestrial polygonal patterns were compiled for polygon side-frequency distributions and these are presented in Fig. 4b. Slowly cooling basalt is reported to possess equant polygonal patterns¹⁰⁻¹². Although the most famous columnar basalt occurrences exhibit a roughly Voronoi polygon distribution, they tend to have an excess frequency at the modal value¹³. Further, basalt, cooling in less than ideal conditions for the formation of polygons, tends to have a mode of five rather than the classic six. Rapidly cooling, basalt-glass crusts on lava lakes tend to have a different character¹⁴. Although they are grossly polygonal and have a large number of five- and six-sided polygons, they have a large number of three- and four-sided members as well. Concoidal fractures are common, and this characteristic undoubtedly plays a part in the observed side-frequency distribution. It is often difficult to define polygonal forms at all under these circumstances. Mud crack networks^{6,12,15-19}, and tensional fractures over salt domes²⁰ typically follow a Voronoi distribution as do contractional thrust ridges in playa salt crusts²¹. Ice wedges and patterned ground are also Voronoi distributed^{7,22}. The common feature of these examples is their formation in isotropic or near isotropic stress conditions, which is not surprising as the ideal hexagonal pattern maximizes the reduction of strain energy per unit surface area by cracking and is therefore the most efficient pattern given an isotropic stress field (see ref. 7).

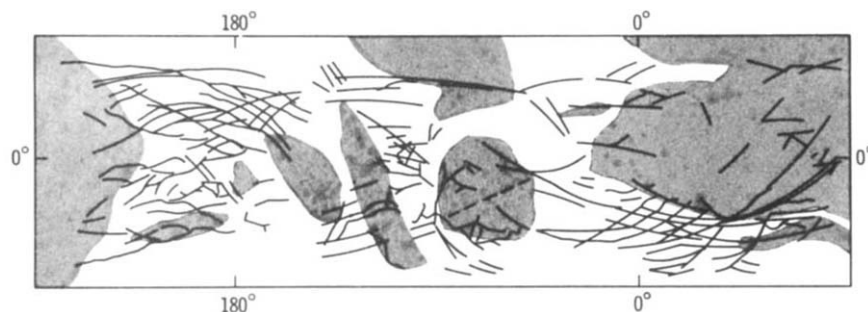


Fig. 1 Global map of lineament patterns on Europa between roughly $\pm 30^\circ$ latitude. Sub-jovian point on the planet is located at lat 0° , long 0° and the anti-jovian point at lat 0° , 180° long. This map is a cylindrical projection, was compiled from low resolution full-disk images from Voyager cameras. Shaded areas correspond to low albedo regions as seen in Voyager blue-filter images. The absence of many apparent lineaments in these areas may be due to either a lack of contrast or to a lack of lineament development in these areas.

The Poisson distribution characterizes randomly intersecting lines in a plane or on a sphere⁶. Statistics for various fracture and joint patterns are presented in Fig. 4c (refs 23, 24). All follow a left-skewed Poisson-type distribution. The most random fracture patterns, as expected, follow the Poisson distribution quite well; however, fracture patterns from strongly oriented stress fields tend towards having orthogonal intersections, thus resulting in left-skewed distribution but with great excess in frequency over the Poisson expectation value at the mode of 4.



Fig. 2 A computer-enhanced and mercator projected mosaic of Voyager 2 images (resolution up to 4.5 km/lp) taken through the blue filter, in which about 25% of the European surface is shown in this mosaic. The anti-jovian point is near the dark, intensely lineated region at mid-left. Map coordinates of the upper right corner, lower right corner, and lower left corner are respectively: lat $+65^\circ$, long 130° ; lat -70° , long 130° ; and lat -70° , long 240° .

If European lineaments were expressions of fracturing in the crust of Europa, then it would be logical to compare the statistics of polygons formed by lineament intersections with the statistics for various terrestrial fracture patterns as outlined above. The fundamental distinction between fracture patterns with a left-skewed (Poisson-type) side-frequency distribution and those with a mildly right-skewed (Voronoi-type) pattern is the presence of 'triple junction' fractures. In an isotropic stress environment (tensional or compressional) failure tends to create nonorthogonal intersecting fractures with intersection angles of about 120° (see ref. 21 for examples of compressional isotropic failure). These triple junctions tend to occur as part of five- and six-sided polygons. The more isotropic and uniform the stress, the more likely it will be that six-sided polygons form. For Europa it is important to determine whether structure exists in the pattern of myriad intersecting lineaments. Preferred orientations or coherent structure in the lineament patterns has implications for the organization of stresses in Europa's crust, if the fracture hypothesis is correct.

An apparently qualitative coherence to at least part of the lineament pattern has been reported^{1,2,25,26}. Parmentier *et al.*²⁷ attempted to analyse the orientations of various lineament groupings and compare these trends with stress fields predicted by tidal stress models²⁸. We have analysed the polygonal patterns²⁹ to find out whether there are statistical differences in the side-frequency distributions between polygons resulting from the intersections of the various lineament types, and if so, what those differences imply about the state of formational stress in the surface of Europa.

Preliminary analysis of three major polygon patterns are reported here:

- (1) The polygons formed by the intersections of Type 3 lineaments.
- (2) The polygons formed by the intersections of Type 1, Type 2, Type 4 and Type 5.
- (3) The roughly equant polygons formed by the intersections of Type 1 and Type 2 lineaments.

The side-frequency distribution for polygons formed by Type 3 lineaments (Fig. 4d) are similar to those found in complex faulted terrain on the Earth, particularly in areas where stresses are well oriented. They have a mode of 4, are left-skewed and exhibit a Poisson-like frequency distribution. The maximum principal stress is apparently oriented roughly north-south as can be seen from lineament orientation²⁷. Nothing can be inferred from the plan pattern alone about the sign of the stress in this case. Some models, however, suggest that it may be compressive^{27,28}.

Polygons formed by the intersections of Types 1, 2, 4, and 5 lineaments occur around the anti-jovian point on Europa. Their overall distribution is not grossly different than the polygons formed by Type 3 lineaments with one important exception: the number of five- and six-sided intersection polygons mapped for the Type 1, 2, 4 and 5 lineaments exceeds those seen in the Type 3 distribution by a factor of 2 and a factor of 12, respectively. That is, there is a significant shift in the distribution to the right (Fig. 4d).

The 'wedge-shaped lineaments' referred to elsewhere^{2,25,26}, and called Type 2 lineaments here, have been cited as evidence

for a nascent type of plate tectonics on Europa²⁶. The presence of numerous 'triple junctions' or three-cornered fractures associated with the intersections of Type 1 and Type 2 lineaments supports this line of speculation. Alternatively, they may be the result of local expansion/contraction phenomena. Regardless of their potential connection with possible plate movements, the Type 2 lineaments, when they are mapped separately from other lineaments, have a grossly equant polygonal pattern (Fig. 5). The side-frequency distribution associated with the polygons formed by Type 1 and 2 (Fig. 5) lineament intersections is shown in Fig. 4d. It appears to be much more right-skewed with a mode of 5, similar to the Voronoi-like distributions seen in tensional

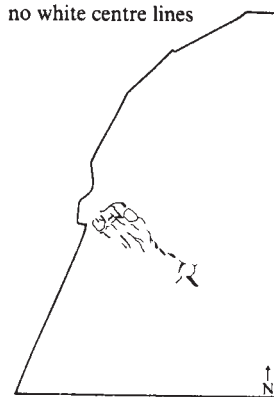
Table 1 Descriptions and locations of lineament types

Lineament type	Location of best examples	Description
1	lat -55° to equator, long 160°	175-500 m long, regular spacing, trend ENE, soft appearance, disrupt and are disrupted by Type 2. Together, Types 1 and 2 form the generally equant equatorial polygonal features discussed in text.
2	lat -50° to equator, long between 160° and 210°	Short wedge-shaped markings; typically straight; form triple junctions; together with Type 1 lineaments they form equant polygonal features around anti-jovian point; darkest of the lineaments in Voyager blue filter; typically trend NW-SE.
3	lat +30°, long 180°	Smooth arcuate features; appear to lie on great circles; longest and most continuous of the lineaments; white medial stripe; remarkable continuity over large distances; intersect at shallow ($\leq 30^\circ$) angles or are parallel; several tens of km wide; cross dark terrains of high and low albedo.
4	two main groups: lat +30°, long 165°; lat -30°, long 160°	Two main groups, but may be ubiquitous in light terrain; faint; reticulate patterns; linear; intersect at high angles; occur in remarkably parallel doublets which maintain constant separation (50 km) over distances of hundreds of km; narrowest of lineaments (5-10 km); subdivide polygons formed by the intersections of Types 1, 2, and 3; seem to cut Type 3 lineaments and are cut by Types 1 and 3; intersect Type 3 lineaments at high angles (80°).
5	lat 0° to -40°, long 165° to 210°	Very short (< 10 -20 km in length); subdivide equant polygons formed by Types 1 and 2 to the limit of resolution; ubiquitous around anti-jovian point.
6	lat -60°, long 150-210°	IAU <i>flexi</i> ; complex series of cusped ridges lying on a small circle crudely centred on the anti-jovian point and the complex of Types 1, 2, and 5 lineaments; appear as distinct ridges with a regular cycloid pattern ² ; one set convex towards the anti-jovian point, the other convex away; arranged in radial pattern around a possible impact crater (may be artefact of position with regard to sub-solar point).
7	lat -40°, long 180-220°	Rare; only two visible examples; may be a modification of Types 1 or 3; extremely high albedo; smooth, continuous and linear; very fresh appearance; not cut by other lineaments; one instance of darkening at intersection with another lineament.
8	lat -65°, long 120°; lat 55°, long 120°	Possibly low sun facing scarps of indeterminate heights ² although Soderblom (personal communication) claims they are albedo markings only; roughly parallel to trend of nearby <i>flexi</i> .

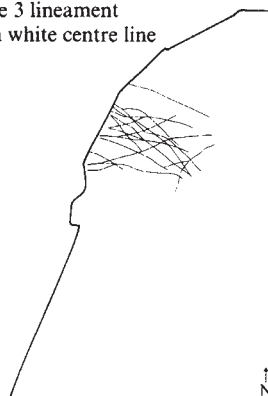
Type 1 lineament
some with centre lines



Type 2 lineament
no white centre lines



Type 3 lineament
with white centre line



Type 4 lineament
no centre line



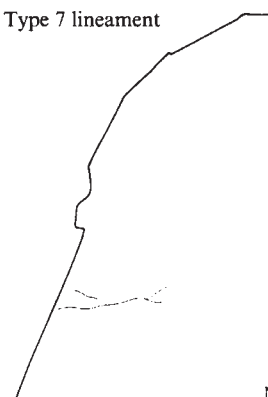
Type 5 lineament



Type 6 lineament



Type 7 lineament



Type 8 lineament
scarps

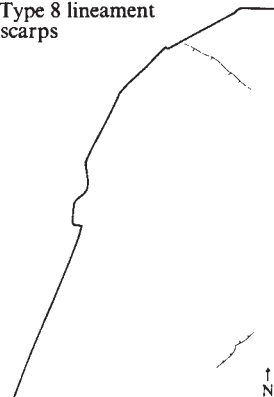


Fig. 3 Sketch maps showing the geographic positions and planimetric morphology of the various types of lineament features observed on Europa. The map outline is the limit of visibility shown in Fig. 2.

fracture patterns. Again, however, the pattern cannot provide information on the sign of the imposed stress; however, isotropic or near-isotropic tension is the predominate factor in terrestrial examples of this style of failure.

Conclusions and discussion

Qualitative and quantitative observations of lineament and polygon patterns on Europa enable several conclusions to be drawn.

First, definite classes of lineaments and their associated polygons have been found on Europa which are separable both by qualitative and quantitative analysis. Second, these classes form distinct groups—often localized, but also some of global character. Finally, these groups have separable geometrical and transectional characteristics, probably related to the stress fields in which they were formed.

These conclusions lead to several speculations. First, the several morphological classes and scales of lineaments may imply different processes acting at different scales, and may also be related to a temporal evolution of the lineament forming processes as suggested by Soderblom²⁵. Further, geographically diverse areas on Europa may respond differently to lineament

formation, which may be construed as evidence for heterogeneity in the European crust. Classification schemes proposed so far^{1,2,25,29} indicate a diversity of lineament type and thus, a complex geologic history.

Second, the observations of roughly equant five- and six-sided polygonal patterns near the anti-jovian point argue for a grossly uniform (probably tensional) stress field around the anti-jovian point on Europa. The uniform tension at the anti- and sub-jovian points may be consistent with tidal eccentricity stresses⁵, however, preliminary calculations (B. Bannerdt, personal communication) point to very low (<10 bar) stresses from this mechanism. Other models invoking internally generated stresses (such as convection, and dehydration of interior) are being pursued^{3,4} and suggest much higher stress levels in Europa's crust. Further, the areal density of the lineament patterns and associated polygons varies with position. Overall, the spacing of tensional polygons varies with material strength and/or rate of stress imposition⁷. Closely spaced reticulate patterns occur at the anti-jovian point and may be indicative of higher imposed stress, of a weaker (or possibly thinner) surface layer, or both.

A more detailed morphological/time-history lineament classification scheme, detailed morphological and geological

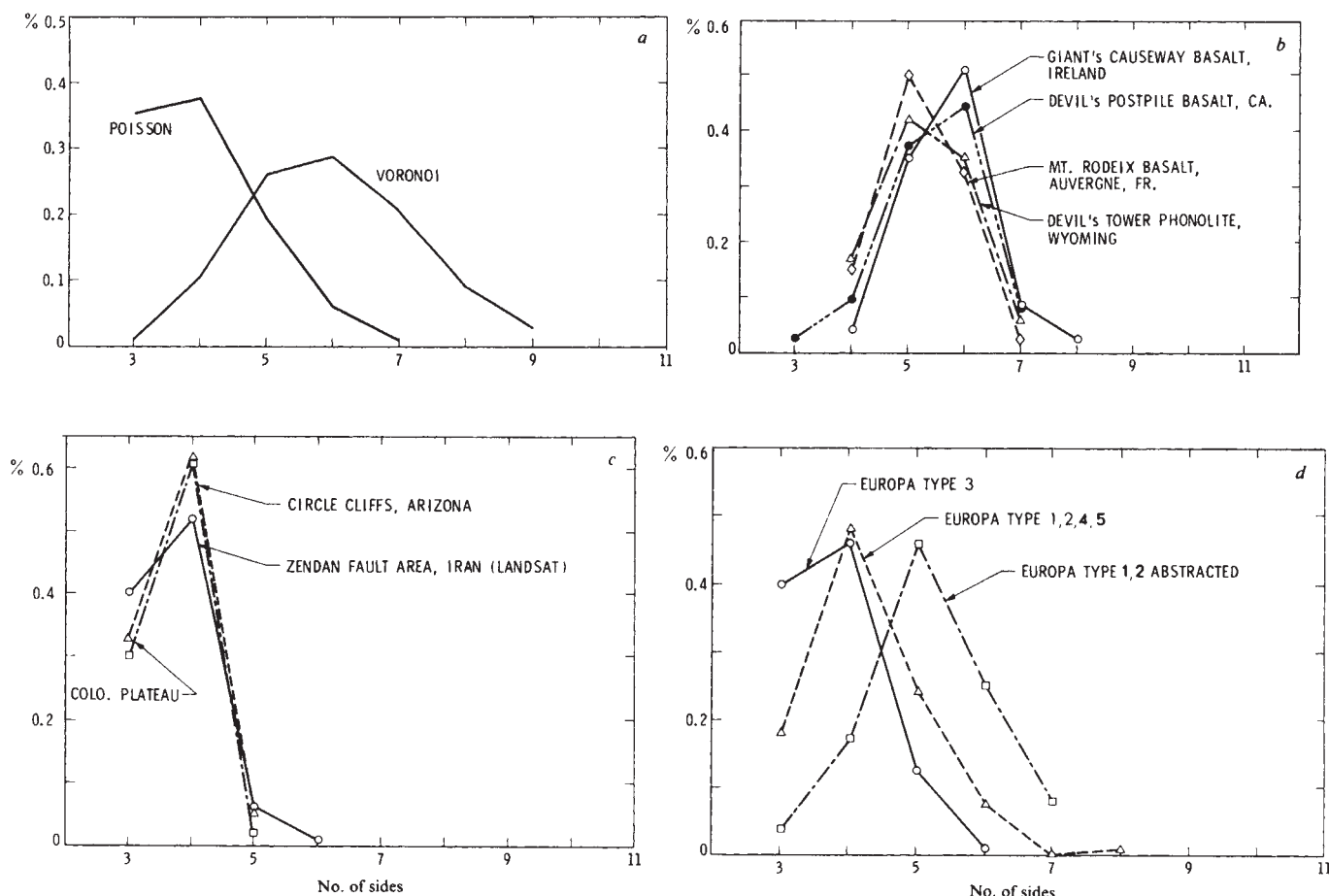


Fig. 4 *a*, Theoretical frequency distributions for polygons of a given number of sides formed by randomly intersecting lines (Poisson) and tessellation polygons formed, for instance by cracking mud (Voronoi) (adapted from Tables 2.1 and 2.2 of ref. 6). *b*, Measured side-frequency distributions for polygons formed by tensional cracking in columnar basalts at well-known localities, compiled from Beard¹³. Note that modes of both 5 and 6 sides are prevalent. The most pristine examples, having formed in presumably ideal conditions (slow cooling rates, few edge effects) have frequency modes of 6. *c*, Side-frequency distributions for intersecting faults in complexly faulted terrain in three areas on the Earth. All show an excess over the random Poisson distribution at the mode. The examples from Circle Cliffs, Arizona and from near the Grand Canyon are in areas where predominate fracture trends are nearly orthogonal, hence the extreme enhancement over the Poisson expectation value. The Zendan fault area in Iran is part of the Zagros crush zone where faults are predominately parallel to the axis of the collision zone and tend to intersect at shallower angles than the other two examples. Nevertheless, there is enhancement at the mode over the Poisson expectation value, however, it is about 10% less than in the orthogonal systems. *d*, Side-frequency distributions for polygons formed by various lineament types on Europa.

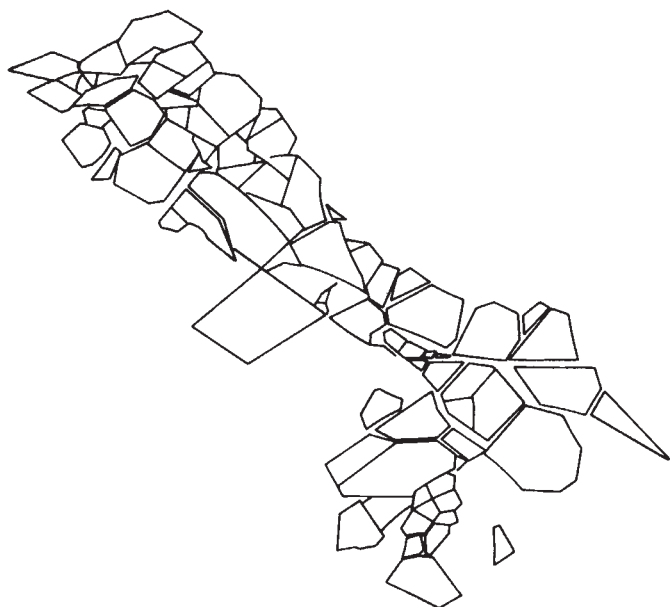


Fig. 5 Sketch map of polygons formed by the intersection of Type 1 and Type 2 polygons on Europa. This region is centred at about lat -20° , long 190° .

studies bearing on lineament formation mechanisms^{3,26}, and more comprehensive statistical analysis of patterns are needed both to confirm observed pattern structure, and to be used as an alternative and quantitative classification tool. Ultimately the existence of lineament patterns must be accommodated within,

and may provide insights for, models of the structure and evolution of both the interior and surface of Europa.

I thank Ken Collerson, Tony Finnerty, Mike Malin, Dennis Matson, Mark Parmentier, Larry Soderblom, and Gary Ransford for useful discussions. This research was carried out while I was a NASA-National Research Council Resident Research Associate at the Jet Propulsion Laboratories of the California Institute of Technology.

Received 5 August; accepted 8 December 1980.

1. Smith, B. A. *et al.* *Science* **206**, 927-950 (1979).
2. Malin, M. C. *IAU Colloq.* No. 57 (1980).
3. Finnerty, A. A., Ransford, G. A., Pieri, D. C. & Collerson, K. D. *Nature* **289**, 24-27 (1981).
4. Ransford, G. A., Finnerty, A. A. & Collerson, K. D. *Nature* **289**, 21-24 (1981).
5. Helfenstein, P., Parmentier, E. M. & Head, J. W. *IAU Colloq.* No. 57 (1980).
6. Crain, I. K. in *Random Processes in Geology* (ed. Meriam, D. F.) 3 (Springer, New York, 1976).
7. Lachenbruch, A. H. *Mechanics of Thermal Contraction Cracks and Ice Wedge Polygons* (Waverly, Baltimore, 1962).
8. Goudsmit, S. *Rev. Mod. Phys.* **17**, 321-322 (1945).
9. Miles, R. E. *Proc. natn. Acad. Sci. U.S.A.* **52**, 1157-1160 (1964); *Math. Biosci.* **6**, 85-127 (1970); *Sankhya, Ser. A* **33**, 145-174 (1971).
10. James, A. V. G. *J. Geol.* **28**, 458-469 (1920).
11. Hewes, L. I. *Am. J. Sci.* **246**, 138-149 (1948).
12. Nelson, R. A. *AAPG Bull.* **63**, 2214-2232 (1979).
13. Beard, C. N. *Bull. geol. Soc. Am.* **70**, 379-382 (1969).
14. Peck, D. L. & Minakami, A. *Bull. geol. Soc. Am.* **79**, 1151-1166 (1968).
15. Kindle, E. M. *J. Geol.* **25**, 135-144 (1917).
16. Kindle, E. M. *Am. J. Sci.* **5**, 329-330 (1923).
17. Longwell, C. R. *Am. J. Sci.* **15**, 135-145 (1928).
18. Lang, W. B. *Science* **98**, 583-584 (1943).
19. Neal, J. T. & Motts, W. S. *J. Geol.* **75**, 511-525 (1967).
20. Badgely, P. C. *Structural and Tectonic Principles* (Harper and Row, New York, 1965).
21. Christiansen, F. W. *Science* **139**, 607-609 (1963).
22. Washburn, *Bull. geol. Soc. Am.* **67**, 823-866 (1956).
23. Iranpanah, A. & Esfandiari, B. *Photogram. Engng Remote Sens.* **46**, 225-229 (1980).
24. Shoemaker, E. M., Squires, R. L. & Abrams, M. J. *NASA Tech. Rep.* 32-1597 (1975).
25. Soderblom, L. A. *IAU Colloq.* No. 57, (1980).
26. Schenk, P. M. & Seyfert, C. K. *EOS* **61**, 286 (1980).
27. Parmentier, E. M., Helfenstein, P. & Head, J. W. *Abstr. 11th Lunar Sci. Conf.* 860-862 (1980).
28. Melosh, J. *Icarus* **31**, 221 (1977).
29. Pieri, D. C., Ransford, G. A. & Collerson, K. *IAU Colloq.* No. 57 (1980).

Europa's petrological thermal history

G. A. Ransford*, A. A. Finnerty* & K. D. Collerson†

* Space Sciences Division, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91103

† Research School of Earth Sciences, Australian National University, Canberra, ACT 2600, Australia

The thermal evolution of Europa is described by a new path of geophysical development. Petrological considerations suggest that heat may have been transported within Europa by sub-solidus convection, and that a large fraction of Europa's water content is retained as hydrated silicates.

WE have reconsidered the thermal evolution of Jupiter's second galilean satellite, Europa, from its accumulation from circum-jovian debris until the formation of surface features imaged by Voyager. By including the petrological sequence that probably accompanied Europa's thermal evolution, a path of geophysical development which differs from previous studies¹⁻³ is indicated. This path gives a present geophysical state that seems to be more in concert with the Voyager observations than earlier models. The petrological considerations imply that heat transport within Europa by sub-solidus convection might have been important throughout much of that satellite's history. We also suggest that a large fraction of the water of Europa is retained in the interior in the form of hydrated silicates.

Thermal history

All previous studies of Europa's thermal history have assumed that the satellite was a mixture of ice and silicates after assembly from the primordial circum-jovian nebula. In these models, Europa is heated by the decay of long-lived radioactive isotopes,

and heat is internally redistributed by conduction. High temperatures are reached rapidly, resulting in a differentiation episode. Silicates are segregated into a core of some 1,450 km radius, with a liquid water mantle of 75-100 km thickness, and a 40-km thick ice crust at the surface^{3,4}. Cassen *et al.*⁵ have studied further evolution from this state within the ice crust and water mantle due to tidal heating effects, and they have concluded that, if once formed, there are conditions in which the water mantle can remain liquid indefinitely. Schubert *et al.*⁶ have argued, however, that solid state convection in an ice crust is likely to remove heat from a liquid water mantle sufficiently quickly to cause freezing of the mantle. Cassen *et al.*⁷ have improved the earlier calculations, and confirm that persistence of a liquid water mantle is very unlikely.

The total amount of water required to dilute the density of a lunar or Io type body ($\rho = 3.5$) to Europa's value ($\rho \approx 3.0$) is ~5 or 6 wt%, compared with 35 or more wt% for Ganymede and Callisto. Such a small percentage of water need not have started in Europa as ice, because there are many hydrated minerals that

Table 1 Weight composition and cation proportions of minerals on Europa

	Weight proportions			Cation proportions (4-oxygen basis)		
	(1)	(2)	(3)	(4)	(5)	(6)
SiO ₂	33.1	35.94	35.40	0.947	1.018	1.000
MgO	23.5	25.52	25.65	0.997	1.071	1.083
FeO	35.5	38.54	38.95	0.848	0.911	0.917
Al ₂ O ₃	2.4			0.080		
CaO	2.3			0.070		
Na ₂ O	1.1			0.061		
NiO	1.9			0.043		
Total	100.0	100.0	100.0	3.043	3.000	3.000

(1) Average composition (wt%) of principal components of Type 1 carbonaceous chondrites on a C-, S- and H₂O- free basis, from ref. 33.

(2) Chondritic composition of column (1), normalized to SiO₂ + MgO + FeO = 100.0.

(3) Calculated composition (wt%) of an intermediate olivine with 46 mol% Fe-end member.

(4) Cation proportions for the chondritic composition in column (1).

(5) Cation proportions for (1), normalized to Si + Mg + Fe = 3.

(6) Cation proportions for intermediate olivine in column (3).

could be stable in a relatively cool planet of chondritic composition (for example, brucite ~30 wt% H₂O, serpentine ~14 wt% H₂O, chlorite ~12–15 wt% H₂O, talc ~5 wt% H₂O, micas ~2–4 wt% H₂O, amphiboles ~2 wt% H₂O). We consider the possibility here that water was present in Europa as water of hydration from the start.

Water of hydration hypothesis

There are two reasons which indicate that Europa might have started its thermal evolution with most, if not all, of its water in the form of water of hydration. The first is a consideration of the likely pressure–temperature conditions in the Europa zone of the circum-jovian nebula during the condensation of the material that eventually becomes the satellite. Fanale *et al.*³ reconstructed the circum-jovian nebula in an attempt to understand the simultaneous presence of ice on Ganymede and the 3.0 density of Europa, taking into account the possible earlier luminosity of Jupiter. Whether Io condensed from the circum-jovian cloud without H₂O, or H₂O was subsequently lost from Io, all reconstructions that give high temperatures for the condensation conditions at Io's orbit result in conditions of high humidity and moderate temperatures at Europa's distance. In such conditions, hydrous silicates may condense stably (reviewed in ref. 8).

Accretional heating during the formation of Europa could have dehydrated these condensation products as they accumulated, but most accretion scenarios lead to the opposite conclusion. Harris⁹ has shown that the matter accumulated into the galilean satellites had to be predominantly circum-jovian in origin for them to have survived their accretion. Ransford *et al.*¹⁰ discussed how Jupiter completely dominates the dynamics of its circum-jovian swarm of planetesimals, making the problem of accretion in circum-jovian orbit different from accretion in circum-solar orbit. The growing planets exert sizeable perturbations on objects within the circum-solar swarm, which 'pump up' random velocities, and make for relatively high velocity collisions between the forming planets and circum-solar planetesimals. In the circum-jovian accretion case, the forming satellites do not randomize velocities, because of the overwhelming close presence of Jupiter, and relative velocities between circum-jovian planetesimals and the forming satellites are characteristic of the difference in velocity between two objects in nearby orbits. These velocities are much smaller than those in the circum-solar accretion cases, and the attendant heating is expected to be much less. In addition, the accumulation of the small bodies in circum-jovian orbit is thought to have

been a moderate time scale process of 10⁵–10⁶ yr or longer^{9,11}, which would not raise temperatures inside Europa to above the dehydration temperature for moderate to low temperature accreting material. Thus, the hydrous minerals formed at the condensation stage were probably not dehydrated by the accumulation process.

The second reason for considering water of hydration contrasts with the first. If the circum-jovian nebula considerations cited above are not true, (if Europa does form as a mixture of ice and silicates), subsequent thermal evolution will melt the ice, which will then hydrate the surrounding silicates, forming minerals such as serpentine, brucite and chlorite. These reactions would occur in the low-to-moderate temperature environment that is presumed for Europa's interior early in its thermal evolution provided the appropriate bulk composition is present within the satellite.

If a volatile-free chondritic bulk composition (+5 wt% H₂O) is assumed for Europa, the anhydrous components are equivalent in composition to 92 wt% olivine with 45 wt% (64 mol%) Mg-end member (forsterite) (Table 1). With the presence of 5 wt% H₂O, therefore, reactions in which olivine is hydrated to give serpentine + brucite or chlorite + brucite are most appropriate to Europa. The phase equilibria for two such reactions from Kitahara *et al.*¹² are reproduced in Fig. 1. A curve representing the stability of clinocllore, a chlorite of composition Mg₅Al₂Si₃O₁₀(OH)₈ using data from refs 13, 14, is also plotted in Fig. 1. The presence of Fe, and of phases other than clinocllore, probably lowers the dehydration temperature of the chlorite by perhaps up to 200 °C. A curve for the maximum stability of brucite¹⁵ is also shown. For comparison, pressure at the centre of Europa is calculated to be ~31 kbar.

The role of Fe in the stability of serpentine and chlorite is not well known for this bulk composition. Serpentine in terrestrial occurrences generally contain <1.5 wt% Fe₂O₃ or FeO (ref. 16). Chlorite in terrestrial occurrences generally contains some Al, while Fe²⁺/Fe³⁺ + Mg covers the complete range 0–1. In the temperature range below 600 or 700 °C it is likely that either immiscible Mg-rich and Fe-rich serpentines, Mg-rich serpentine + Fe-rich chlorite, or an intermediate chlorite with no serpentine, will coexist with brucite throughout the pressure range within Europa. Alternatively, the Fe component may crystallize to magnetite or metal, depending on oxygen fugacity. Such high density materials could separate to form a core.

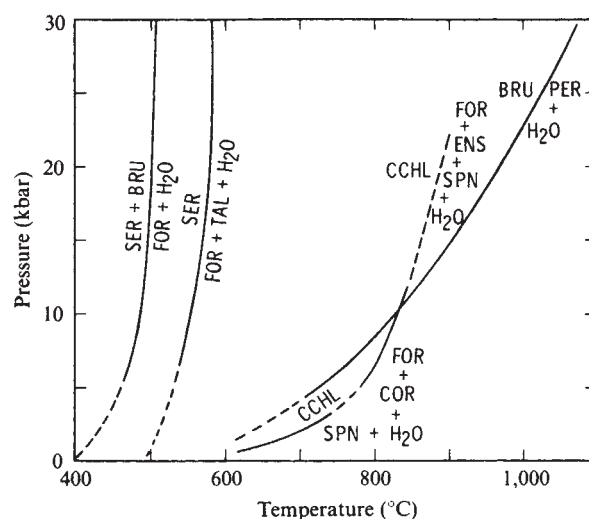


Fig. 1 Stability curves for hydrous minerals and assemblages. Data for forsterite (FOR), serpentine (SER) bulk compositions from ref. 17; clinocllore (CCHL) bulk composition at pressures above 3 kbar from ref. 13; CCHL below 3 kbar from ref. 14; brucite (BRU) composition from ref. 15. Curves are dashed where extrapolated. Other minerals talc (TAL), cordierite (COR), spinel (SPN), enstatite (ENS), periclase (PER).

The products of the hydration reactions represented in Fig. 1 contain 11.7–16.1 wt% H₂O, with 13.7 wt% for the 'chondritic' intermediate olivine composition of Table 1. Using the latter percentage as a likely value for chondritic composition, the entire 5 wt% H₂O in Europa would be consumed if reaction went 36.4% to completion. If water is evenly distributed within Europa, the interior would consist of about two-thirds olivine and one-third serpentine and/or (chlorite + brucite). Alternatively, all the water could be concentrated in an outer hydrated layer composed of serpentine and/or chlorite + brucite, having a thickness of ~270 km.

Discussion

While the water of Europa may once have been incorporated in the interior of the satellite as hydrous silicates, it is not obvious that the water currently exists in the interior after 4,500 Myr of thermal evolution. Using the Moon as an analogue, because its radius is nearly the same as that of Europa, current thermal models employing convective heat transport and materials properties thought to be appropriate for the Earth and the Moon^{17–19} predict temperatures well above those at which hydrous minerals dehydrate over most of the interior (Fig. 2). The lithospheres in the models of Fig. 2 are ~300–400 km thick. In a convective Moon model with initial post-accretion temperature of 1,300 °C, Schubert *et al.*²⁰ calculate a lithospheric thickness of 550 km after 4,500 Myr. While Europa probably started at a much lower initial post-accretion temperature, a planetary object's thermal state after 4,500 Myr has been shown^{17,20} to be more dependent on the rheology of the planetary interior than on initial temperature. The upper 200–300 km of the thermal boundary layers or lithospheres in the models cited above are at temperatures below the 500–700 °C dehydration temperatures. Therefore, the hypothesis that most of the water of Europa is incorporated in a relatively cool lithosphere composed of hydrous minerals, which is situated above a hot convecting interior of anhydrous silicates, is consistent with our current knowledge of planetary thermal evolution.

An alternative model may be considered. Tozer^{21,22} has suggested that water of hydration affects silicate material properties by decreasing the resistance to creep, that is, by decreasing the effective viscosity. In his convection models for the Moon, the term for temperature dependence of assumed newtonian viscosity was scaled to the solidus temperature of hypothetical rocks. Because water lowers the solidus temperature of basalt, the viscosity of the hydrous model Moon is lowered by a factor of ~20, and consequently convection can take place at temperatures below 500 °C. A thermal profile for a Moon composed of "water saturated basalt" from Tozer's work is incorporated in Fig. 2. If such a model is applied to Europa, it is clear that hydrated silicates can remain stable throughout the interior, because the low-temperature convection transports heat at a rate sufficient to maintain the interior below the dehydration temperatures.

While "it is generally accepted that subsolidus creep is negligible at temperatures below about 800 °C"²⁰, and therefore that hydrous minerals are unstable in regions that are convecting, this prediction is based on values of materials properties for anhydrous silicates. Hydrolytic weakening of silicate minerals has been convincingly demonstrated only for quartz²³, despite the work of Blacic²⁴ on olivine. The presence of water weakens the rocks under uniaxial compression, including the serpentine²⁵ and chloritite²⁶ of special interest for our model. However, this weakening is probably a pore-pressure effect²⁷ and is likely to be ineffective at high pressures (>10 kbar) where pore volume is expected to be negligibly small. Additional information on the rheology of 'wet' serpentinite and chloritite will be needed before the possibility of convection within hydrated mineral assemblages can be properly evaluated.

It is expected that some free water gains access to the surface during this evolution, but not in vast quantities. Most of the water present in Europa can be retained in the hydrated

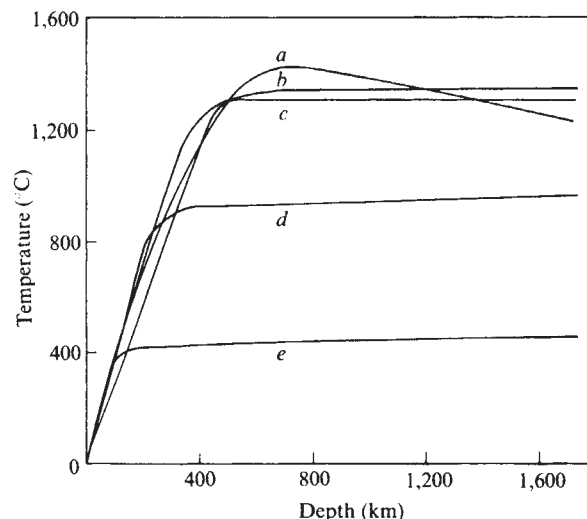


Fig. 2 Spherically averaged temperature–depth curves for the Moon. Data for a, ref. 34; b, ref. 19; c, ref. 18; d, ref. 22 (anhydrous dunite model); e, ref. 22 (water-saturated basalt model).

minerals in the interior. We predict, therefore, that the ice layer on the surface of Europa is much thinner than the 150 km that had been estimated before the Voyager mission. A thin ice crust on Europa has important implications for tidal coupling and surface morphology (such as cracking—see refs 28, 29 for more details).

The geophysical state that results from a convective evolution agrees with the basic observations about Europa. The synchronous rotation of the satellite would result soon after the subsolidus convection began because of the triaxiality that such patterns could induce in its body³¹. Tidal energy dissipation could involve the entire satellite, as opposed to the crust and core arrangement of the earlier estimates of the thermal history⁵. The completely solid Europa described here would remove a heuristic concern about the earlier Europa—what are the dynamics of a crust with almost no coupling to the core during a tidal interaction? Do they interact with Jupiter independently and librate with respect to each other?

The cracking patterns on Europa's surface suggest some sort of global deformation mechanism^{28,32} and internal convective activity would certainly provide such deformation. This aspect will be discussed in more detail by Finnerty *et al.*²⁹. The distortions of the silicate surface that are created by the areas of upwelling and subduction in the convective model can fracture the overlying thin layer of ice, generating crack patterns which can be tested against the observed patterns for various orders of convective activity.

Conclusions

We have argued that most of Europa's complement of water can be locked up in the form of hydrated silicates in a thick convective boundary layer or possibly even throughout most of the body of Europa. The water of hydration is in the minerals chlorite and/or serpentine, and brucite, and solid state convection probably keeps a sufficient volume of the planet at temperatures below the dehydration temperatures for these minerals to incorporate all the water needed to explain the density of Europa. With most of the water tied up in hydrated silicates, the ice layer at Europa's surface is probably quite thin, perhaps only a few kilometres.

We thank D. L. Matson, F. P. Fanale, R. J. Phillips, S. Daly, E. Ivins and D. Pieri for helpful discussions. This work represents one phase of research carried out at the Jet Propulsion Laboratory, California Institute of Technology, under contract NAS 7-100, sponsored by NASA.

Received 5 August; accepted 8 December 1980.

1. Lewis, J. S. *Science* **172**, 1127–1128 (1971).
2. Consolmagno, G. J. & Lewis, J. S. *Icarus* **34**, 280–293 (1978).
3. Fanale, F. P., Johnson, T. V. & Matson, D. L. in *Planetary Satellites* (ed. Burns, J.) 379–405 (1977).
4. Smith, B. A. *et al. Science* **206**, 927–950 (1979).
5. Cassen, P., Reynolds R. T. & Peale, S. J. *Geophys. Res. Lett.* **6**, 731–734 (1979).
6. Schubert, G., Stevenson, D. J. & Ellsworth, K. *Icarus* (submitted).
7. Cassen, P., Peale, S. J. & Reynolds, R. T. *Geophys. Res. Lett.* (submitted).
8. Grossman, L. & Larimer, J. W. *Rev. Geophys. Space Phys.* **12**, 71–101 (1974).
9. Harris, A. W. *Icarus* **34**, 128–145 (1978).
10. Ransford, G. A., Matson, D. L. & Johnson, T. V. *EOS* **60**, 307 (1979).
11. Cameron, A. G. W. & Pollack, J. B. in *Jupiter* (ed. Gehrels, T.) 61–84 (1976).
12. Kitahara, S., Takenouchi, S. & Kennedy, G. C. *Am. J. Sci.* **264**, 223–233 (1966).
13. Fawcett, J. J. & Yoder, H. S. Jr *Am. Miner.* **51**, 353–380 (1962).
14. Chernosky, J. V. Jr *Am. Miner.* **59**, 496–507 (1974).
15. Irving, A. J., Huang, W. L. & Wyllie, P. J. *Am. J. Sci.* **277**, 313–321 (1977).
16. Deer, W. A., Howie, R. A. & Zussman, J. *Rock-forming Minerals* Vol. 3 (Wiley, New York, 1962).
17. Turcotte, D. L., Cooke, F. A. & Willeman, R. J. *Proc. 10th Lunar planet. Sci. Conf.* 2375–2392 (1979).
18. Schubert, G., Young, R. E. & Cassen, P. *Phil. Trans. R. Soc. A* **285**, 523–536 (1977).
19. Cassen, P. *et al. Phys. Earth planet. Inter.* **19**, 183–196 (1979).
20. Schubert, G., Cassen, P. & Young, R. E. *Icarus* **38**, 192–211 (1979).
21. Tozer, D. C. *Phys. Earth planet. Inter.* **6**, 182–197 (1972).
22. Tozer, D. C. *The Moon* **9**, 167–182 (1974).
23. Kirby, S. H. & McCormick, J. W. *Bull. miner.* **102**, 124–137 (1979).
24. Blacic, J. D. *Flow and Fracture of Rocks* (eds Heard, H. C., Borg, I. Y., Carter, N. L. & Raleigh, C. B.) 109–115 (American Geophysical Union Geophysical Monogr. 16, 1972).
25. Raleigh, C. B. & Paterson, M. S. *J. Geophys. Res.* **70**, 3965–3985 (1965).
26. Murrell, S. A. F. & Ismail, I. A. H. *Tectonophysics* **34**, 207–258 (1976).
27. Patterson, M. S. *Experimental Rock Deformation—The Brittle Field* (Springer, New York, 1978).
28. Pieri, D. *Nature* **289**, 17–21 (1981).
29. Finnerty, A. A., Ransford, G. A., Pieri, D. & Collerson, K. D. *Nature* **289**, 24–27 (1981).
30. Runcorn, S. K., *Proc. R. Soc. A* **296**, 270–284 (1967).
31. Roberts, P. H. *Mathematika* **12**, 128–137 (1900).
32. Helfenstein, P. & Parmentier, E. M. *Proc. 11th Lunar planet. Sci. Conf.* (submitted).
33. Ringwood, A. E., *Composition and Petrology of the Earth's Mantle*, 192 (McGraw-Hill, New York, 1975).
34. Turcotte, D. L., Hsui, A. T., Torrance, K. E. & Oxburgh, E. R. *J. geophys. Res.* **77**, 6931–6939 (1972).
35. Schubert, G. *Ann. Rev. Earth planet. Sci.* **7**, 289–343 (1979).

Is Europa surface cracking due to thermal evolution?

A. A. Finnerty*, G. A. Ransford*, D. C. Pieri* & K. D. Collerson†

*Space Sciences Division, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91103

†Research School of Earth Sciences, Australian National University, Canberra, ACT 2600, Australia

We propose that some of the surface features of Europa may have originated by processes within a model planet in which hydrated silicates are stable.

THE images of Europa obtained from Voyager display remarkable curvilinear albedo features that range in scale from the limits of resolution to truly global. It has been proposed that most of these features (the lineae) are fractures, and that some (the flexi) are ridges^{1–6}. We point out that some surface features of Europa may have originated by processes within a model planet in which hydrated silicates are stable, as proposed by Ransford *et al.*⁷. Because we deal with only a few types of the features that have been observed, additional processes, or perhaps entirely different processes than proposed here, may be operating. Because of the paucity of data bearing on remote planetary objects, however, our understanding of these processes can be advanced only by constructing successively more realistic models that can be tested against the limited observations.

In the Ransford *et al.*⁷ model for Europa, a region of convecting anhydrous silicates is overlain by a lithosphere, the upper 200–300 km of which is composed of hydrous minerals. The ice crust need not be thick (not nearly the 75–150 km of ice and possibly water in pre-Voyager models) because all the H₂O needed to explain the lower density of Europa compared to Io can be contained within the hydrated silicates. To relate surface features of Europa to this model, we first examine the mechanical properties of hydrated silicates and ice.

Mechanical properties

The compressive strengths and ductilities of serpentinite, of partially serpentinitized peridotite, and of chloritite have been measured at confining pressures up to 6.6 kbar at various temperatures^{8,9}. Complex but consistent behaviour was observed. At temperatures below the dehydration temperatures (500–700 °C) serpentinites and chloritites are about as strong as granite, and brittle, becoming more ductile as confining pressure and temperature increase. As temperatures approach the dehydration temperatures, the hydrous rocks weaken slightly, but at the dehydration temperatures, strengths decline drastically to <500–1,000 bar and the specimens become much more brittle. This is apparently due to release of free water, because at temperatures below the dehydration temperatures, the intro-

duction of free water causes similar weakening and embrittlement. This behaviour has been related to some types of faulting on Earth^{8–10}. If free water is present in the lithosphere of Europa, therefore, fracturing should occur at quite low stress levels.

The mechanical behaviour of water ice is very similar to that of anhydrous silicates. At a small fraction of its melting temperature it deforms in a brittle fashion¹¹, while at a high fraction of melting temperature it deforms plastically by a creep mechanism (reviewed in ref. 12). The mechanical behaviours of thin and thick ice crusts on Europa should consequently differ. (We do not consider a liquid water mantle for Europa because new calculations¹³ suggest that the previously proposed liquid zone¹⁴ is very unlikely.)

Thickness of the ice crust

Using a near-surface (below diurnal influences) temperature for Europa of 93 K (ref. 15), and a thermal gradient of 3 K km⁻¹ (from the conductive region of the thermal models discussed in ref. 7), a temperature of 60% of the melting temperature of ice will be reached at ~24 km depth. If such a relative temperature is taken as marking the brittle-to-ductile transition, then any ice existing below 24 km depth will deform by plastic rather than brittle mechanisms. While such deep ice will be capable of transmitting isotropic stresses and allow shallower brittle ice to fracture, any nonisotropic stress from below will be compensated by lateral flow within warm ice. The presence of a reticulate or polygonal fracture pattern in the anti-jovian region of Europa⁶ on a scale larger than the 150 km maximum depth of an ice layer, but on a smaller than planetary scale, suggests that nonisotropic stresses have propagated through the ice crust and that the crust is consequently thinner than about 24 km, at least in the anti-jovian region. Smith *et al.*¹ have also suggested that the ice layer may be thin (<50 km) based on the implications of two terrain types and very low surface relief. Therefore, we will consider the ice crust to be thin and brittle, acting as a recorder of stresses imposed on the underlying hydrated silicate mantle.

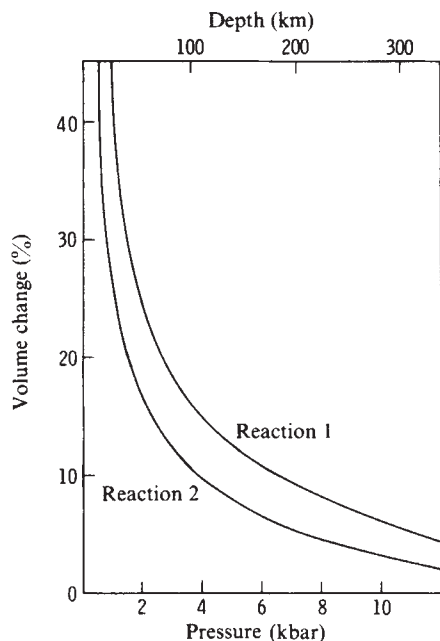


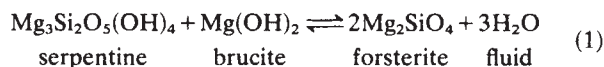
Fig. 1 Relative volume changes as a function of depth for dehydration reactions involving serpentine.

Models for two sets of surface features on Europa have been developed: (1) the long, relatively dark lineaments that lie on great and small circles, and frequently possess bright medial stripes (principally the Types 1 and 3 lineaments of ref. 6); (2) the complex reticulate network of fractures in the anti-jovian region, surrounded by the series of cusped ridges defining a small circle about 60° outside the anti-jovian point (Types 5 and 6 lineaments of ref. 6).

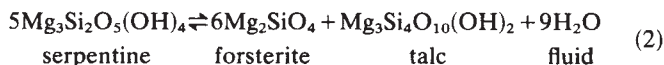
Global scale lineaments

The great lengths (>1,000 km in many cases) and distribution (for example, Asterius Linea defines a nearly complete great circle) require a global scale mechanism for formation of Types 1 and 3 lineaments. A possible mechanism is planetary expansion. Volume changes due to thermal expansivity can be positive or negative depending on the thermal history (see ref. 16). Dehydration of hydrated silicates at relatively low pressures, however, results in large positive volume changes, and may be responsible for formation of Types 1 and 3 lineaments.

Dehydration may be represented by reactions such as:



and



Other reactions involving iron-bearing components and/or chlorite lead to similar conclusions. Considering a closed system in which the total H_2O cannot vary, the change in volume for reactions (1) and (2) may be calculated from the data summarized in Table 1. The molar volumes of the minerals do not change dramatically with temperature and pressure, and in any case these changes approximately cancel in calculating the change in volume, so their room temperature, one atmosphere volumes are used. For H_2O , however, the molar volume does change drastically with temperature and pressure. The dehydration temperature of serpentine is $\sim 500^\circ\text{C}$, and is nearly independent of pressure. Therefore, volume changes for reactions (1) and (2) are calculated as a function of pressure at 500°C and plotted in Fig. 1. A depth scale for Europa is also illustrated. Molar volume data for H_2O at pressures >10 kbar are not available, but the volume probably does not decrease below

$14.4 \text{ cm}^3 \text{ mol}^{-1}$ at 30 kbar and 500°C . This corresponds to volume changes at the centre of Europa that are close to zero.

If Europa accreted at low temperatures⁷, hydrated minerals would initially be distributed throughout the satellite. The temperature at a given depth would increase with time due to decay of radioactive isotopes. Assuming an adiabatic or superadiabatic temperature gradient, the hottest location is at the centre of the planet, regardless of whether solid state convection commences. At some time the temperature at the centre of the planet would rise above $500\text{--}700^\circ\text{C}$, and dehydration would commence. A dehydration front would coincide with the upward evolution of the $500\text{--}700^\circ\text{C}$ isotherm. If H_2O did not escape upwards, the assemblage of anhydrous minerals + vapour would expand relative to the original hydrated assemblage.

If H_2O can percolate upwards, it will react with anhydrous minerals in the layer above the $500\text{--}700^\circ\text{C}$ isotherm to increase the hydrous mineral content from about 40% to as high as 100%. Any excess H_2O will migrate still higher and hydrate additional layers. In this case, the dehydrated region decreases in volume while the layer of increased hydration expands. Because ΔV is greater with shallower depth, however, the net volume change is positive.

This expansion creates stress in the overlying hydrated silicates, and at the same time the dehydration process provides the free water that completes the hydration of these rocks. Eventually, the water is concentrated in a completely hydrated layer extending from the surface to $\sim 270 \text{ km}$ depth⁷. Additional water percolating from below can no longer be bound in hydrated minerals, and thus weakens and embrittles these rocks even though they are below their dehydration temperatures. The hydrated silicate shell may be modelled as a spherical pressure vessel, in which tangential and radial stresses due to dehydration are at maximum at the inner surface and minimum at the outer surface. For a fracture to propagate to the surface, the crack probably needs to be filled with water, provided by the dehydration, as it grows. As Anderson and Grew¹⁷ noted, water is particularly effective at promoting crack growth in silicates by stress corrosion.

The presence of bright medial stripes within lower albedo lineaments hundreds of kilometres long and tens of kilometres wide must be explained by any hypothesis of the origin of these features. Malin² noted that the bright 'cores' are in places hundreds of metres above the surrounding terrain. Also, we note that whereas the white medial stripe is usually very uniform in width within a particular lineament, the darker portion is

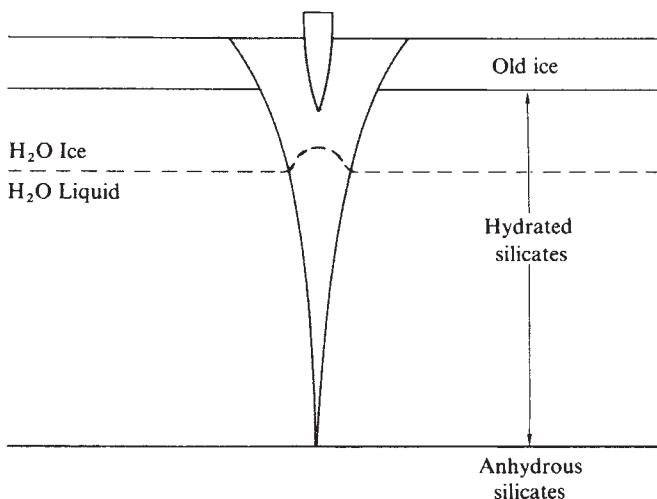


Fig. 2 A representation in cross-section of the formation of planetary scale dark lineaments with bright medial stripes. The shaded region is xenolith-rich H_2O 'magma'. The dashed line represents the freezing temperature of ice. The wedge at the top is new ice extruded upwards as the freezing isotherm migrates downward during thermal re-equilibrium of the magmatic column.

Table 1 Molar volumes of components ($\text{cm}^3 \text{mol}^{-1}$)*

Serpentine	$\text{Mg}_3\text{Si}_2\text{O}_5(\text{OH})_4$	108.5
Brucite	$\text{Mg}(\text{OH})_2$	24.63
Forsterite	Mg_2SiO_4	43.786
Talc	$\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$	136.25
Vapour	H_2O	18.169†

* Mineral data from Robie *et al.*²⁶.† Data from Burnham *et al.*²⁷ for H_2O vapour at 500 °C, 10 kbar.

quite variable and, where it widens on one side of the medial stripe, there is frequently a corresponding widening on the opposite side.

We suggest the following explanation (Fig. 2), analogous perhaps to CO_2 -driven kimberlite eruptions on Earth. When a crack is initiated at the base of the lithosphere, its propagation upwards is aided by stress corrosion as water from the dehydrating region fills the crack. Because this aqueous fluid is highly corrosive to Si–O–Si bonds and of low viscosity, the crack tip can propagate rapidly¹⁷. Relatively high fluid velocities may be attained, in which case xenoliths can be broken from the sides of the crack and incorporated into the ascending fluid. As the surface is approached, velocity of the fluid will diminish due to loading in the 'magmatic' column and loss of heat to the walls of the conduit. A breccia of frozen water and xenolithic silicates and ice, of lower albedo than the surrounding icy crust, will fill the upper reaches of the conduit, while in the lower portion H_2O will remain liquid and xenoliths can settle out. As the crack propagates laterally, presumably following a great circle, eruptions of this brecciated material with varying vigour will cause variations in the surface width of the low albedo material. At the conclusion of this process, the isotherm coincident with the water-ice transformation will be bowed upwards within the 'breccia pipe'. As this isotherm evolves downwards within the fracture towards thermal equilibrium, additional water will freeze, causing expansion and a new stress. Because the depth of ice breccia in the crack will be least at the centre of the crack, a second stage fracture will form within the ice breccia and in the middle of the initial crack. New ice will extrude along the new fracture, rising above the surrounding terrain due to the liquid-ice volume change. Inasmuch as the new ice is essentially xenolith-free, it will have higher albedo than the ice breccia on either side.

Features about the anti-jovian region

A concentration of fractures interpreted as tensile features, in polygonal or reticulate patterns has been noted^{4,6,18} over a region near the anti-jovian point on Europa. This interpretation, based on an analysis of the frequency distribution of polygon sides, has been justified by Pieri⁶. The polygonal pattern is formed by the intersections of Types 1 and 2 lineaments and is subdivided to the limits of resolution by the Type 5 lineaments of Pieri. For this modelling exercise, we also assume that these are fractures formed in a tensional environment.

Because of their geometrical relationship to the tensional fractures in the anti-jovian region, we consider the flexi, or Type 6 lineaments of Pieri in any model of processes occurring in this locality. These cusped ridges appear to surround the tensile fractures in a ring about 60° out from the anti-jovian point, and have up to several hundred metres of relief. Their maximum extent is not known because of incomplete high-resolution coverage and unfavourable lighting angles. There is no recognized evidence of erosional or magmatic processes on Europa to form these ridges. If they are of tectonic origin, they probably formed within a region of compressional stress. Strom *et al.*¹⁹ described lobate scarps and ridges up to 500 km long and 3 km high on Mercury, uniformly distributed over the hemisphere imaged by Mariner 10, and attributed their formation to crustal compression during thermal contraction of Mercury. While the distribution of European cusped ridges differs from that on Mercury, and we do not yet know the profiles of the ridges, we

feel that the interpretation as compressional tectonic features is most likely. In the absence of data to the contrary, we assume that the compressional features formed contemporaneously with the tensional features discussed above.

We are faced, then, with a broad region of extension centred near the anti-jovian point, surrounded by a region of compression. Based on the radial distribution of tensional features, doming, and concentration of volcanism in the Tharsis area of Mars, Carr²⁰ suggested that a region of upwelling within a convecting mantle was centred under Tharsis. Solomon and Chaiken¹⁶ rejected this interpretation because no regions of compression corresponding to zones of return flow are apparent on Mars. Although this model may not work for Mars, for Europa, we suggest that a region of upwelling in the convecting anhydrous silicate interior, surrounded by a region of return flow, may have been located beneath the hydrated silicate mantle at roughly the anti-jovian point during the formation of the tensile and compressional features. The lineaments formed above the convection cell by brittle deformation within the hydrated silicate lithosphere.

We still need to explain the location of the convective upwelling at the anti-jovian region. This is probably not accidental, because the redistribution of hot, low density and cold, high density material by convective processes alters the moments of inertia of the satellite, and can thus influence the orientation of the satellite when it achieves orbital period-spin rate lockup with the primary.

In combination with the mass distribution associated with equatorial bulging due to rotation, a second-order convection pattern would cause the triaxiality needed for the tidal locking of the rotation period of Europa. This process has been discussed extensively in connection with heat transfer and nonhydrostatic shape of the Earth's Moon (see refs 21–23), and results in diametrically opposed zones of upwelling becoming aligned with the radial to the primary. In the case of the Moon, the maximum shear stress imposed by the nonhydrostatic figure is probably insufficient to fracture the lithosphere²⁴, and a dynamic process such as convection is not required. For Europa, the 'wet' hydrated silicate lithosphere should be far weaker than the probably anhydrous lithosphere of the Moon, and consequently a dynamic process such as convection should be revealed by failure of the lithosphere.

A second-order convection pattern requires a small core, as might have been provided in Europa by separation of iron or magnetite on deserpentinization⁷. Such a pattern is shown in Fig. 3. The distribution of extensional and compressional fea-

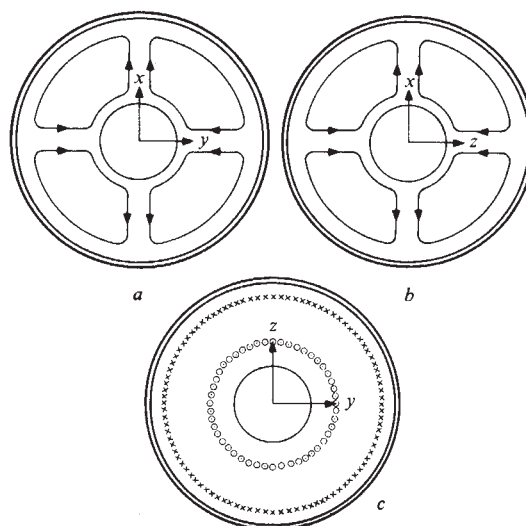


Fig. 3 Idealized second-order convection patterns in three mutually orthogonal projections. Axis of rotation is the z-axis, direction towards Jupiter is along the x-axis, and the y-axis points in the instantaneous direction of motion of Europa in its orbit around Jupiter. ○, Upwelling; ×, downflow.

tures in the anti-jovian region of Europa is remarkably similar to the locations of zones of upwelling and return flow in Fig. 3c. However, the effects of other processes may be superimposed on the surface of Europa, and convection within a planetary interior is probably more complicated than second order. Nonetheless, a major second-order component may be present.

Discussion

A spherical pressure vessel model for Europa predicts no preferred orientation for the planetary-scale fractures. Helfenstein and Parmentier¹⁸ have noted, however, a tendency for these lineaments to intersect with an angle of 60–70°. They use the elastic shell theory developed by Melosh²⁵ to show that the patterns of these lineaments and the location of a region of tension in the anti-jovian region are consistent with relaxation of a triaxial figure composed of two biaxial distortions: an equatorial bulge and a bulge along the radial to Jupiter. They propose a mechanism in which the equatorial bulge relaxes as Europa despins due to tidal interactions, and the anti-jovian bulge relaxes as the orbit of Europa recedes from Jupiter. We are not convinced that such a preferred orientation of global scale fractures exists on Europa because of limited photographic coverage, uncertainty about which fractures were studied by Helfenstein and Parmentier, and the probability that fractures

formed by several different processes may have been included in their analysis. There are also two difficulties with their model: the magnitude of stresses imposed by relaxation of the figure of Europa (tens of bars) probably does not exceed the strength of the lithosphere (Banerdt, personal communication); and the tidal bulge cannot have been located in the anti-jovian region until after Europa had despun into a tidally-locked configuration. If preferred orientations of global scale fractures are documented, we suggest that global expansion due to dehydration of the interior provided stresses great enough to fracture the lithosphere, and that superimposed stresses due to tidal despinning may have influenced the orientation of these fractures. The triaxial contribution to the fracture patterns may have been provided by dynamic loading of the lithosphere by convection in the interior.

We re-emphasize the hypothetical nature of our arguments. We hope that these speculations will stimulate new observations and will lead to new models that will improve or supersede the model proposed here.

We thank D. L. Matson, E. Ivins, S. Daly, B. Banerdt, F. P. Fanale and R. J. Phillips for helpful discussions; and D. L. Matson, F. P. Fanale for helpful comments. This research represents one phase of research carried out at the Jet Propulsion Laboratory, California Institute of Technology, under contract NAS 7-100, sponsored by NASA.

Received 5 August; accepted 8 December 1980.

1. Smith, B. A. *et al. Science* **206**, 927–950 (1979).
2. Malin, M. C. *IAU Colloq. No. 57*, abstr. 7–2 (1980).
3. Helfenstein, P., Parmentier, E. M. & Head, J. W. *IAU Colloq. No. 57*, abstr. 7–4 (1980).
4. Parmentier, E. M., Helfenstein, P. & Head, J. W. *Abstr. 11th Lunar Planet. Sci. Conf.* 860–862 (1980).
5. Pieri, D., Ransford, G. & Collerson, K. *IAU Colloq. No. 57*, abstr. 7–3 (1980).
6. Pieri, D. *Nature* **289**, 17–21 (1981).
7. Ransford, G. A., Finnerty, A. A. & Collerson, K. D. *Nature* **289**, 21–24 (1981).
8. Raleigh, C. B. & Paterson, M. S. *J. geophys. Res.* **70**, 3965–3985 (1965).
9. Murrell, S. A. F. & Ismail, I. A. H. *Tectonophysics* **31**, 207–258 (1976).
10. Heard, H. C. & Rubey, W. W. *Bull. geol. Soc. Am.* **77**, 741–760 (1966).
11. Parameswaran, V. R. & Jones, S. J. *J. Glaciol.* **14**, 305–315 (1975).
12. Weertman, J. in *Physics and Chemistry of Ice* (eds Walley, E., Jones, S. J. & Gold, L. W.) 320–337 (Royal Society of Canada, Ottawa, 1973).

13. Cassen, P., Peale, S. J. & Reynolds, R. T. *Geophys. Res. Lett.* (submitted).
14. Cassen, P., Reynolds, R. T. & Peale, S. J. *Geophys. Res. Lett.* **6**, 731–734 (1979).
15. Gaffney, E. S. & Matson, D. L. *Icarus* (submitted).
16. Solomon, S. C. & Chaiken, J. *Proc. 7th Lunar Sci. Conf.* 3229–3243 (1976).
17. Anderson, O. L. & Grew, P. C. *Rev. Geophys. Space Phys.* **15**, 77–104 (1977).
18. Helfenstein, P. & Parmentier, E. M. *Proc. 11th Lunar planet. Sci. Conf.* (submitted).
19. Strom, R. G., Trask, N. J. & Guest, J. E. *J. geophys. Res.* **80**, 2478–2507 (1975).
20. Carr, M. H. *J. geophys. Res.* **79**, 3943–3949 (1974).
21. Roberts, P. H. *Mathematika* **12**, 128–137 (1965).
22. Runcorn, S. K. *Proc. R. Soc. A* **269**, 270–284 (1967).
23. Cassen, P., Young, R. E. & Schubert, G. *Geophys. Res. Lett.* **5**, 294–296 (1978).
24. Phillips, R. J. & Ivins, E. R. *Phys. Earth planet. Inter.* **19**, 107–148 (1979).
25. Melosh, H. J. *Icarus* **31**, 221–243 (1977).
26. Robie, R. A., Hemingway, B. S. & Fisher, J. R. *Bull. U.S. geol. Surv.* **1452** (1978).
27. Burnham, C. W., Holloway, J. R. & Davis, N. F. *Geol. Soc. Am. Spec. Pap.* **132** (1969).

Sex ratio evolution in a variable environment

E. L. Charnov*, R. L. Los-den Hartogh†, W. T. Jones‡ & J. van den Assem†

* Department of Biology, University of Utah, Salt Lake City, Utah 84112

† Zoölogisch Laboratorium, Rijks Universiteit Leiden, Leiden, The Netherlands

‡ Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

We develop a natural selection model for sex ratio control in a spatially variable environment. Predictions of sex ratio alteration as a function of environmental change are tested in laboratory experiments with two parasitic wasps. Field data from a variety of other organisms also support the model. Finally, we discuss possibilities and difficulties for testing this type of evolutionary model.

SEX allocation theory deals with the impact of natural selection on the allocation of resources to male compared with female reproductive function¹. For dioecious species the problem of interest is the evolution of the sex ratio; for a simultaneously hermaphroditic species, it is the allocation to sperm versus eggs in each breeding season^{2–5}; for a sequential or sex-reversing hermaphrodite, the questions are the sex order (male or female first?) and the proportion of an individual's lifetime devoted to each sex^{6–12}. The theory also gives the conditions for a sexual state to be evolutionarily stable. For example, it specifies when some form of hermaphroditism is favoured over dioecy^{1–16}.

It was previously¹ shown that many sex allocation problems have the property that the equilibrium or ESS¹⁷ condition satisfies a particular optimality principle. The allocation to male versus female function, which is favoured by natural selection, can often be shown to maximize the fitness gains through male function multiplied by the gains through female function¹. From this product formalism, one can derive the classic results of sex

ratio theory. Examples are the 'equal resource into each sex' theorem of Fisher¹⁸, the 'local mate competition' results of Hamilton¹⁹ and several results from the Trivers–Hare theory for eusocial Hymenoptera^{20,21}. Here, we shall apply the formalism to sex ratio evolution in a variable environment and will provide a set of laboratory tests for the resulting predictions. As the organisms which stimulated our modelling efforts were parasitic wasps, we shall develop the model with reference to their life histories.

Parasitic wasps in a variable environment

Imagine an outcrossing parasitic wasp which has discrete generations and where the females attack hosts over a wide range of sizes^{22–34}. A single egg is laid on each host. The host is paralysed or killed by a sting from the female wasp, so that the total food for her offspring's development is contained in the host at the moment of attack. If the host is small, the wasp larva will have relatively little food and will emerge as a small adult. If

the host is large, the resulting wasp will be large. Suppose further that the reproductive consequences of being a large compared with a small adult wasp differ, depending on whether the individual is a male or a female. For example, over her lifetime, a large female may lay 10 times as many eggs as a small female; however, a large male may be only three times as effective as a small male at inseminating females. As the female wasps lay eggs in a variety of host sizes, the environment is thus variable with respect to opportunities for production of sons versus daughters. The mother is assumed to control her sex ratio (proportion male eggs) as a function of host size. Haplodiploid sex determination provides her with a physiological mechanism for this control. Our interest is in predicting what sex ratio she will produce in a given host size. To do this, we first specify the host size distribution and the expected reproductive success for an adult wasp of a given sex and size.

Let $f(x)$ be the probability density function for hosts of size x which are attacked by the female. For simplicity, assume x to be continuous. Let $W_1(x)$ be the relative fitness of a son derived from a host of size x . A male's fitness is measured relative to other males¹, so that $W_1(x)$ scales the ability of a male of size x to inseminate females over his lifetime. Let $W_2(x)$ be the lifetime egg production of a female derived from a host of size x . Both these fitness measures include survivorship to adulthood, which may vary with host size²². Finally, let $r(x)$ be the proportion of unfertilized or male eggs laid in host size x . $r(x)$ is controlled by the mother wasp. Using techniques from population genetics, one can show that the equilibrium or ESS¹⁷ $r(x)$ satisfies the following product relationship¹. It is the function, $r(x)$, which maximizes the product of the fitness gained through the decisions to produce sons and that gained through the decisions to produce daughters, or:

Maximize

$$\left[\int_x f(x) W_1(x) r(x) dx \right] \times \left[\int_x f(x) W_2(x) (1 - r(x)) dx \right] \quad 0 \leq r(x) \leq 1 \quad (1)$$

If we impose the condition that a large daughter gains more in relative fitness than a large son [$W_2(x)/W_1(x)$ increases with x], the solution to equation (1) reduces to a very simple form^{1,35}. Only sons should be produced in small hosts and only daughters in large ones. There is a certain host size (τ) where the switch from sons to daughters occurs (Fig. 1a). Equation (1) now takes the form:

Maximize

$$\left[\int_0^\tau f(x) W_1(x) dx \right] \times \left[\int_\tau^\infty f(x) W_2(x) dx \right] \quad (2)$$

Although the model predicts a threshold (τ) for the sex ratio change, it is unlikely that biological data would show such an abrupt transition. A more likely pattern would be that of all sons in small hosts and daughters in large, with a gradual sex ratio transition in between (illustrated in Fig. 1a)^{1,35}. We expect a gradual transition for two reasons. First, the model assumes time constant fitness and host distribution values. If these vary through time, then so does τ . The quality of a host may also depend somewhat on factors which cannot be known to the mother when she deposits an egg. Selection would then favour a more gradual shift in sex ratio. However, a second reason is that even if the mother wasp made perfect decisions, the experimenter may not be able to classify the hosts perfectly with respect to criteria used by the wasps. For example, differentiation of equation (2) with respect to τ shows that the hosts are theoretically to be classified by the ratio $W_2(x)/W_1(x)$. Those with low ratios are son hosts, those with large are daughter hosts. Let k be the threshold ratio. Now, suppose that the wasp makes perfect decisions, with all hosts above k being given to daughters. If the experimenter, however, uses a measure of host size ($\hat{x}$) which is imperfectly correlated with the $W_2(x)/W_1(x)$ ratio, we will not see the threshold. The experiment would show a gradual shift in sex ratio as a function of $\hat{x}$, our measure of host quality.

In addition to the sex ratio shift with host size, the model makes one other general type of prediction. The concepts of large and small host as discussed above have no absolute meaning. That is, a host is only large or small relative to the other hosts being attacked. To calculate theoretically whether a host is to be a son or daughter host, we must first know the host size distribution which is being attacked. We expect to find an increasing fraction of daughters in larger hosts; however, what constitutes larger may vary from place to place, or time to time. Consider the host marked z on Fig. 1b. If the distribution of host sizes is i , z is a large host. If the distribution is ii , z is a relatively small host. Host z should contain a greater proportion of daughters when large (case i) than when small (case ii).

Two other factors implicit in the model deserve further comment. First, the sex ratio under discussion is the ratio at the egg stage. Data often show that survivorship declines in small hosts²². If this decline is different for the two sexes, the differential mortality as a function of host size would itself generate a sex ratio shift among emerging wasps, independently of a true sex ratio shift at the egg stage. However, such differential mortality would also contribute to natural selection favouring a shift by the mother wasp. Some evidence indicates that daughters may sometimes be unable to reach pupation size in very small hosts (a pupal parasite of Lepidoptera³⁴), but even here a strong sex ratio shift at the egg stage with host size could be demonstrated. The relativity prediction discussed previously is a good control for

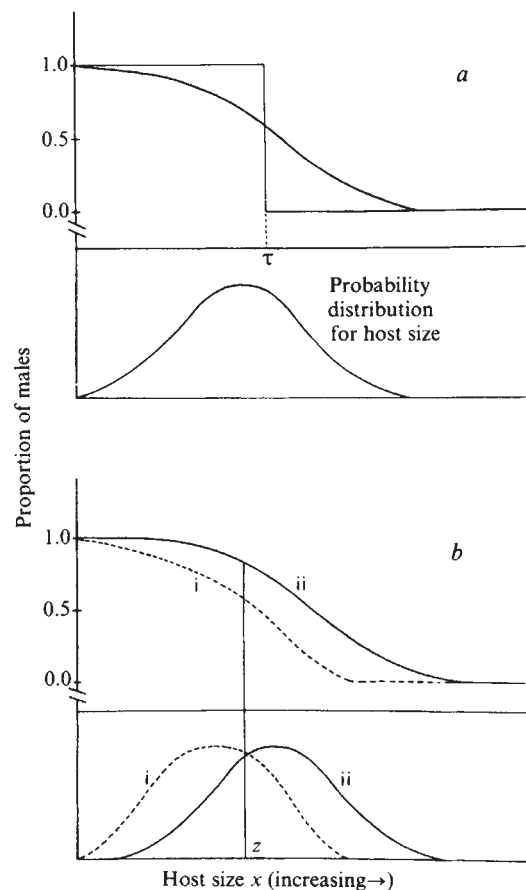
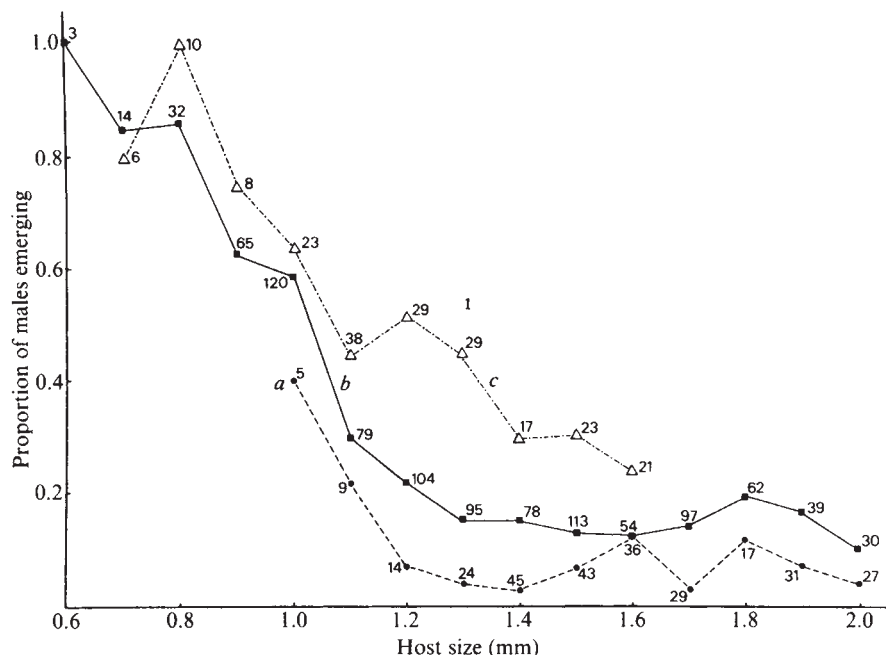


Fig. 1 Sex ratio as a function of host size. *a*, Provided females gain more fitness by being larger than do males (that is, $W_2(x)/W_1(x)$ increases with x), the sex ratio is predicted (equation (2)) to be female biased in large hosts, male biased in small. There is a threshold host size (τ) where a change-over occurs. For reasons discussed in the text, the expected pattern is not a threshold, but more gradual sex ratio shift. *b*, Whether a given host is to be a son host or a daughter host depends on the entire host size distribution. Host z is a relatively large host if the host distribution is i ; it is a small host if the distribution is ii . It should contain more males when it is small. Host size distributions are simply illustrative.

Fig. 2 Sex ratio in *Lariophagus*. The proportion of males emerging is plotted against the size of the hosts (weevil larvae) (tunnel width) which were offered in a 'carousel experiment'. Curve *b* results when female wasps are presented sequentially with 20 hosts of a single size. Curves *a* and *c* result when wasps are presented with an alternating sequence of two host sizes, where the hosts are 0.4 mm different in size. Curve *c* is where the host of interest was the smaller of the two. For example, hosts of 1.4 mm were offered alone and gave a sex ratio of 15% males (curve *b*). When they were offered alternately with 1.8-mm hosts, they gave a sex ratio of 30% males (curve *c*). When offered with 1.0-mm hosts, their sex ratio dropped to 2% males (curve *a*). The number of emerging wasps is given for each data point. The experimental technique is as follows: the weevil *Sitophilus* is cultured on wheat grains at 25 °C. Twice a week freshly emerged weevils are placed on fresh (unfrozen) grain for oviposition and removed again 7 days later. Grains with the necessary age range of larvae can then be irradiated with X rays to measure the exact size of the larvae by measuring tunnel diameter³⁷. The grains are then stored at 10 °C for up to 14 days. At 10 °C the larvae do not grow and only a small percentage die. Grains with larvae of a desired size are picked out and used in carousel experiments. The oviposition carousel (described in ref. 30) makes it possible to offer individual female wasps grains with known contents for a specified period of time in a specified order. Inseminated females are isolated from cultures on the day of their emergence and stored for 24 h at 18–20 °C. They are used in a carousel experiment, with a series of 20 grains. Each grain is offered to a female for 2.5 h, then labelled and stored at 25 °C until a weevil or a wasp emerges. If there is no emergence, the grain is opened to see what happened to the weevil larvae. The head width was measured for all wasps.



this effect, as the shift is predicted within a single host size. The model also predicts that the overall sex ratio should be biased towards males. However, this is again sex ratio at the egg stage. If survivorship declines with host size, then at emergence the overall sex ratio may be female biased (because males are put in small hosts).

The second factor is the assumption that the species is outbred. Hamilton^{19,36} has developed a theory for certain forms of inbreeding, or nonrandom competition among males for mates, which predicts a generally female-biased sex ratio. In the most extreme cases, all females in a brood are inseminated by their brothers. Here, theory predicts (and data strongly support) that a mother wasp should produce mostly daughters. This 'local mate competition' (LMC) is thus a factor which may alter the overall sex ratio in the direction of females. Although we cannot eliminate it as a factor in our wasp systems, it seems unlikely as a general explanation for a host shift in sex ratio. Long-term (~20 yr or 200 generations) laboratory cultures of one species studied here²² consist of hundreds of wasps mixed together. There is much mixing of the offspring of individual females. Such conditions are quite the opposite of those postulated by Hamilton and would select against LMC effects. In this same species (*Lariophagus*), other experiments presented the wasps with groups of hosts and sometimes put two or more female wasps together with a batch of hosts. The present experiments presented a single female with one host at a time and they gave essentially the same results as the former. With a strong LMC model, the wasps would be predicted to behave differently in the two conditions. For these reasons, we have derived the predictions from a model with outbreeding (Fig. 1).

On testing the model

A general test of the model would consist of showing that sex ratio varied as a function of host size, and that the daughter/son fitness comparison also changed with host size. Both would have to change in the predicted directions. We would also have to show that differential mortality or LMC could not account for

the results. The predicted relative nature of the sex ratio shift itself provides a powerful, qualitative test of the hypothesis. There are at least three ways in which we might test this prediction.

(1) Geographical variation. If in different places the wasp species is associated with different host size distributions, this natural variation can be turned into a test. For instance, if the same host is present in both locations, but is a small host in one place and is large in the other, a sex ratio shift is expected in that host.

(2) Laboratory selection experiments. It should be possible to mimic the effects of geographical variation in the laboratory. If the parasitoids can be reared on only small hosts, or only large hosts, sex ratio shifts through time (over several generations) are expected, if the sex ratio is a heritable character. The small hosts should shift from producing mostly males towards a more equal sex ratio. The large hosts should shift from mostly females towards a more equal ratio.

(3) Temporal variation. If the wasps naturally confront host size distributions which change from generation to generation, they might have the ability to alter their sex ratio on a short time scale, in response to the prevailing distribution. The most reasonable source for a shift in host size utilization would be a shift in host abundance. Because small hosts are poorer for offspring, a mother might be expected to ignore them when confronted with an abundance of larger hosts. This behavioural shift would require the wasps to pick up, store and use information about the distribution. However, if the natural host size distribution was either constant through time, or continued to change within a wasp generation, we might expect the wasps not to respond to short-term shifts in the distribution. We would then require geographical variation or a laboratory selection experiment to test the relativity idea.

We report here the results of a series of experiments on this host size model. Our experiments relate to the abilities of two parasitoid species to alter their sex ratio decisions on a short time scale. We tested the basic model by looking at sex ratio as a function of host size. We then confronted wasps with altered

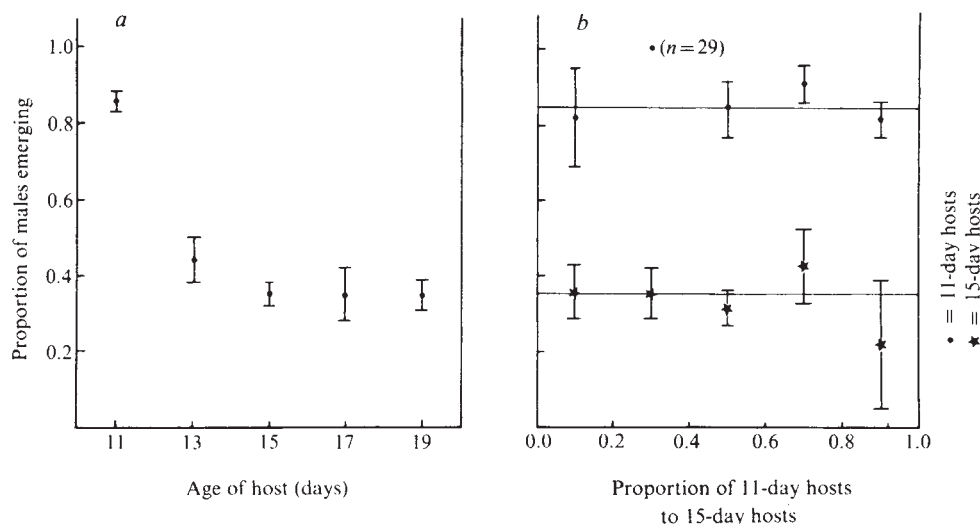


Fig. 3 Sex ratio as a function of host age for *Heterospilis* attacking *Callosobruchus*. **a**, Wasps presented with a single-aged host for a period of 24 h (30°C and 70% relative humidity, host density at ~20 hosts per female wasp). Data given are means ± 2 s.e. **b**, Wasps were in an environment consisting of 11- and 15-day hosts presented simultaneously. In the five experiments, the proportion of 11-day hosts varied from 0.1 to 0.9. Data are the mean sex ratios within each host age (± 2 s.e.). The lines in **b** refer to the proportion of males found in **a**.

host size distributions and measured the sex ratio shift. Our data indicate that large wasps come from large hosts and that both males and females gain increased reproductive success by being large.

Sex ratio in *Lariophagus* and *Heterospilis*

Lariophagus distinguendus is a small (1–3 mm) parasitic wasp (family Pteromalidae) which attacks larvae of the common granary weevil, *Sitophilus granarius*^{22,29,30}. The weevils oviposit in grains of wheat, and a single larva develops inside the grain. The parasite stings the larva and deposits a single egg. An adult wasp emerges about 18 days later (at 25°C). The wasp will parasitize a variety of host sizes²². The larva hollows out the grain as it develops. Size of weevil larvae is correlated with age, but the relationship is imprecise. For the present experiments we measured host size directly. The size of the weevil larva is strongly correlated with the diameter of its tunnel³⁷. Tunnel diameter was measured on X-ray photographs^{22,37}.

Heterospilis prosopoidis is a small (1–3 mm) parasitic wasp in the family Braconidae. It has a similar life history to that of *Lariophagus* in that it lays a single egg on the larvae of weevils, attacking a variety of host sizes. It was studied here attacking the azuki bean weevil, *Callosobruchus chinensis*. At 30°C, weevil development (egg to adult) takes about 21 days. Weevil larvae 11–19 days old are accepted by the wasp. Experiments with this system used weevil age as a measure of host size.

Van den Assem²² had previously shown, using weevil age as a measure of size, that *Lariophagus* adjusts its sex ratio as a function of weevil size. Mostly males were produced in young weevils, mostly females in older weevils. His experiments were carried out by presenting wasps with weevils of only one age class. The experiments were also controlled to show that the observed sex ratio shift could not be due to differential, sex-specific mortality (even though young weevils produced a much higher death rate). The data also suggested that the wasp used a relative measure of host size.

We have repeated these earlier²² experiments, using the more exact measure for host size. Figure 2b shows the results. As expected, sons are produced in small hosts, daughters in large. A second experiment consisted of presenting wasps with two host sizes, offered in equal abundance. The hosts were always 0.4 mm different in size and the wasp was presented with an alternating sequence (large, small, ...). The data from these two experiments thus consist of a given host size in three situations: alone, smaller of two sizes and larger of two sizes. For example, we have 1.4-mm hosts alone, and paired with 1.0- or with 1.8-mm hosts. If we consider some host size z , it is clear that the three experiments (z small, z alone, z large) represent successively more left-shifted host size distribution curves. This corresponds

to a shift from distribution ii to distribution i in Fig. 1b. If the wasp is adapted to alter its sex ratio on a short time scale, it should put relatively more daughters into host z as we move across the three treatments.

Curve *a* in Fig. 2 represents the sex ratio as a function of host size, when the host represented was the larger of the two hosts (for example, 1.0 was paired with 0.6). Note that every point lies below (is more female biased) the curve for the hosts when they were by themselves. Curve *c* (Fig. 2) shows the corresponding relationship for hosts where they were the smaller of the two hosts (for example, 1.0 was paired with 1.4). With a single exception, 0.7 mm, this curve lies above (more male biased) the other two curves. We have not provided statistical tests for the individual host sizes because the hypothesis relates to the general order of the curves. It is indeed remarkable that, even given small (10–30) to moderate (50–100) sample sizes, the curves preserve their theoretically predicted order, over the entire range of host sizes. This is the strongest evidence for the shift predicted by sex ratio theory.

Using host age as a measure of size, one of us (W.J.T.) carried out a similar set of experiments with *Heterospilis*. Presenting wasps with a single host size produced the relationship in Fig. 3a. Small hosts (11 days) were very male biased, whereas larger hosts were more female biased. A series of control experiments showed that this result could not be due to differential, sex-specific mortality. To see if these wasps could also adjust the sex ratio to a shifting host environment, we carried out a second set of experiments with just two host ages (11 and 15 days, or 15 and 19 days), presented in equal numbers. In comparison with the experiments in which the wasp received only one host size, the 11- and 15-day hosts now showed no altered sex ratio. Indeed, the 15-day hosts showed the same sex ratio when alone, or when combined with either 11- or 19-day hosts. Moderate to large sample size (88–1,024 emerging wasps per host size) makes these results quite reliable. Only one predicted sex ratio shift was seen. When 19-day hosts were presented alone, they gave a proportion of males of 0.36 ($n = 786$). However, when they were presented with equal numbers of 15-day hosts, the sex ratio dropped to 0.25 ($n = 88$). Such a shift is statistically significant (one-sided, Fisher exact test; $P = 0.03$) in the predicted direction.

We carried out a further set of experiments with *Heterospilis*. These involved 11- and 15-day hosts, presented to the wasp in various proportions (proportions of 11-day hosts 0.1, 0.3, 0.5, 0.7, 0.9). As can be seen in Fig. 3b, the wasp failed in 8 out of 10 cases to show any deviation (significant at the 0.05 level) from the sex ratio predicted from the experiments with only one host size. Clearly, there is no pattern of sex ratio shift with changing host proportions (at least for 11- and 15-day hosts). Although

Heterospilis does not immediately adjust its sex ratio to host size distributions, it is still possible that its offspring sex ratios are attuned to host distributions in a way predicted by the model. It may be that the host size distributions naturally encountered by *Heterospilis* do not vary greatly between wasp generations or that they vary within generations, and thus these wasps would not be expected to respond to short-term shifts in the distribution. At least for this species, a selection experiment or geographic comparison seems to be necessary to test the model further.

At least two other parasitic wasps which show a sex ratio shift with host size also show short-term adjustment of the sex ratio^{23,24}. Both species overproduce daughters in large hosts, sons in small. Chewyreu²³ studied parasitoid wasps of the genus *Pimpla* (family Ichneumonidae) which attack lepidopteran pupae. When he offered a female wasp a mixture of host pupae consisting of *Sphinx* (large) and *Pieris* (small), the wasp produced mostly females in the large, males in the small. However, when the *Pieris* pupae were alternated with those of *Vanessa* (still smaller), the same wasp produced female offspring in the former and males in the latter. Sandlan²⁴ also studied a pupal parasite of Lepidoptera, the wasp *Coccygomimus turionella*. Here, individual wasps confronted with a single host size gradually altered the sex ratio through time. If the single size was large, the sex ratio declined from being female biased to being more male biased. A small size showed the opposite change—male biased towards more female biased. All these sex ratio shifts are in the theoretical direction.

Fitness as a function of host size

As the sex ratio shift is towards daughters in larger hosts, a further test of the theory consists of determining whether a female gains more fitness by being large than does a male. Measuring individual fitness is very difficult, especially for males, where the value must reflect the relative ability to gain access to females. Using *Lariophagus*, a set of experiments was designed to investigate whether wasp size was correlated with the size of its host, and also whether increased wasp size became translated into increased individual fitness. Figure 4 shows the result of rearing almost 2,000 individuals. Clearly, larger weevils give rise to large wasps. Two facts about Fig. 4 are of particular interest. First, the relationship is different for males and females. From the same-sized host, males are on average smaller than the corresponding female. This presumably reflects the fact that males show a shorter developmental time to adulthood on any given host size²². Second, both curves show a steep linear increase over the weevil size range 0.7–1.3 mm. At 1.3 mm both show an abrupt transition. Average male size no longer increases with weevil size, whereas average female size goes up at a much reduced rate (slope of curve here is about 12% the previous slope).

Figure 5 shows that increased size translates into both increased male and increased female fitness. For females the measure is the lifetime production of eggs, for males it is length of life. This is not a complete measure of male fitness, because it gives no indication of male mating ability. Small males have no difficulty in getting females to accept them as mates²². Because females typically mate once for life, small males would seem to give adequate numbers of sperm, but we do not know how such small males fare in mate competition with larger males.

A similar set of experiments with female *Heterospilis* showed that lifetime fecundity increased about 20 times, comparing 11-day with 15-day hosts [1.3 offspring (s.d. = 2.87, $n = 61$) compared with 27.9 (s.d. = 8.38, $n = 46$)]. Interestingly, males from the same-aged hosts showed a much smaller fitness size effect, the measure here being females inseminated over a 3-h period (with 20 females available). Eleven-day males inseminated an average of 2.8 females (s.d. = 2.91, $n = 38$), compared with 8.3 (s.d. = 2.36, $n = 31$) for 15-day males. Although these data suggest that daughters gain more by being large than do sons, they are not conclusive. It would be very useful to know how individual size affects other fitness

components such as female search ability or female ability to attack large hosts.

There is another type of data which bear on this fitness gain issue, at least for *Lariophagus*. If fitness within each sex is related to the size of the wasp, host sizes over the range 1.3–2.0 mm show very little change in relative female/male size (Fig. 4), whereas relative female/male size is increasing over the host size range 0.6–1.3 mm. We might then expect that host sizes above 1.3 would be treated quite similarly with respect to sex ratio. As shown in Fig. 2b (host size alone experiments), the proportion of sons drops rapidly with increasing host size until a host size of 1.3 mm. The sex ratio is about 0.17 males at this host size, but it does not become more female biased as we move to even larger hosts. The experiments presented in Fig. 2 show that the wasp can alter its sex ratio when a two-host environment consists of both hosts above 1.3 mm. However, these shifts are small relative to the alteration in sex ratio observed over a host size range of 0.6–1.3 mm. The sex ratio versus host age relationship measured earlier by van den Assem²² shows a similar abrupt transition in sex ratio at the host age of 23–24 days. X-ray calibration shows that this age corresponds to a mean host size close to 1.3 mm.

Other hymenopteran systems

There are other parasitoid systems where a range of host sizes is attacked, and where sex ratio alters with host size^{22–34}. The shift is always in the direction of more females from larger hosts. There are other hymenopteran systems which show similar trends. 'Trap nesting' (ref. 38) refers to free-living bees and wasps where the mother places food plus an egg in a cell constructed inside a crevice (for example, a hollowed-out twig). The size of the crevice places constraints on the cell size, the amount of food packed in and consequently the size of the resulting adult wasp^{38,39}. Trap nesters have been much studied because they will commonly nest in soda-straws, holes drilled in wood blocks, or other man-made crevices^{38,39}. Because of the importance of some trap-nesting bee species for agricultural pollination³⁹, many data now exist. Figure 6 shows the sex ratio (proportion of emerging males) as a function of hole diameter in artificial nesting material for 11 species of bees and wasps. With one exception, these data are from the book by Karl Krombein³⁸. This documents no trap nester with a reverse sex ratio shift, although several species show evidence of no sex ratio shift for the hole sizes presented (several wasps of the genera *Stenodynerus* and *Ancistrocerus*). A reverse shift might be expected in species where the males show aggression and territoriality with respect to mating^{35,40}. Here, increased size could

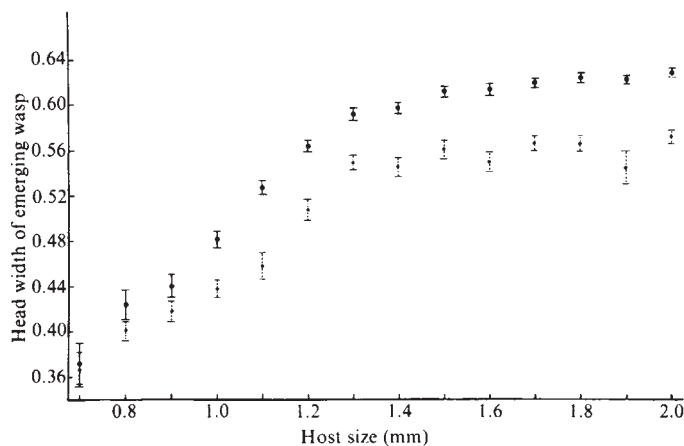


Fig. 4 Size of *Lariophagus* related to its host size (tunnel width). Means (± 1 s.e.) of head width of males (lower curve) and females (upper curve) are plotted against host weevil size. Experimental details are given in Fig. 2 legend. Least squares regressions calculated on the means: (1) δ , host sizes 0.7–1.3 mm, $y = 0.16 + 0.28x$, $r = 0.98$, $P < 0.001$. (2) δ , host sizes 1.4–2.0 mm, correlation not significant at 0.05 level. (3) ϕ , host sizes 0.7–1.3 mm, $y = 0.11 + 0.37x$, $r = 0.99$, $P < 0.001$. (4) ϕ , host sizes 1.4–2.0 mm, $y = 0.54 + 0.044x$, $r = 0.92$, $P < 0.01$.

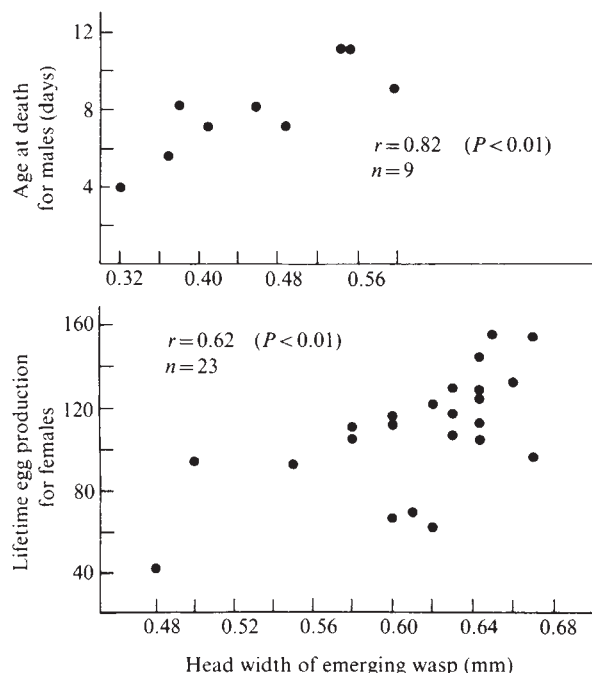


Fig. 5 Fitness as a function of individual size in *Lariophagus*. Both males and females gain by being large. Females were presented with an abundance of hosts. Experiments were run at 18 °C.

be translated into relatively greater male reproductive success³⁵. It would be very interesting to know if any trap-nesting species shows geographical variation in the hole-size distribution. Such variation should correlate with sex ratio shifts within a specified hole size, similar to those predicted (Fig. 1b, host size z) for parasitoids. However, the situation for trap nesters is somewhat more complicated than the simple model developed here. At least two other sex ratio effects occur with the hole-size shifts. There are often several cells placed in the same crevice—that is, a straw will contain a sequence of offspring. Because males typically emerge before females, it is common for the mother to put more daughters in the deep cells, more sons near the opening³⁸. In this way, the sons can get out first without having to destroy the wasp in front. Except where different mothers may supersede each other at a particular hole, the offspring in a given hole will be siblings. Some degree of cooperation may be expected⁴¹, but the parents should benefit by using deep crevices for a mixture of sons and daughters. The other factor affecting sex ratio is that a son costs (in the sense of Fisher^{18,21}) less than a daughter to produce. They require smaller cells (regardless of the diameter of the hole) and less food³⁸. The equilibrium sex ratio should reflect this lowered cost through production of more males^{20,21}. Even though these two factors complicate the situation, the hole-size effect, as illustrated in Fig. 6, still seems to be a dominating factor.

Discussion

Various biological systems in addition to Hymenoptera show sex ratio shifts of the type discussed here. These include pandalid shrimp^{12,42}, various orchids⁴³, entomogenous nematodes^{44,45} and molluscs of the genus *Crepidula*¹¹. In these cases, larger individuals typically become females, smaller ones male. Some of these species are sex reversers (sequential hermaphrodites), beginning life as a male and later changing into a female. Of course, many sex reversers go in the other direction; particularly among coral reef fish, species are known where individuals begin life as females, and become males when they are large enough to compete for a breeding territory^{6-10,46}.

As developed here and elsewhere^{6-12,35,42,47,48}, an understanding of the direction of the shift seems to depend on knowledge of which sex gains relatively more by being large. Whether the problem is the sex ratio to be produced by a wasp constrained to rear a small offspring or the 'choice' to develop into a small adult male or female imposed on a nematode in a crowded host^{44,45}, the principles underlying the force of natural selection remain the same.

Probably the largest gap in our knowledge is a clear demonstration of which sex gains relatively more by being large (except perhaps for some protogynous fish^{6,7,46}, where it seems fairly clear that males gain more). Because of the difficulty in measuring fitness with sufficient precision to test whether females gain more by being large, we propose here an alternative experiment. The data suggest that males also gain by being larger. If a laboratory culture can arrange for females not to gain fitness by being larger, the selection pressures will be to reverse the sex ratio shift. The culture conditions will then favour males in larger hosts, females in smaller.

E.L.C. thanks J. Bull and J. Werren for many years of discussion on problems of sex allocation. Ken Sandlan, J. Bull, Richard Green, J. van Ierse, John Endler and D. Davidson read and improved the paper. E.L.C. and W.T.J. were supported by grants from the US NSF. J. v.d. A. and R.L.L.-d.H. were supported by the Netherlands Organization for the Advancement of Pure Science (ZWO), grant 89-177.

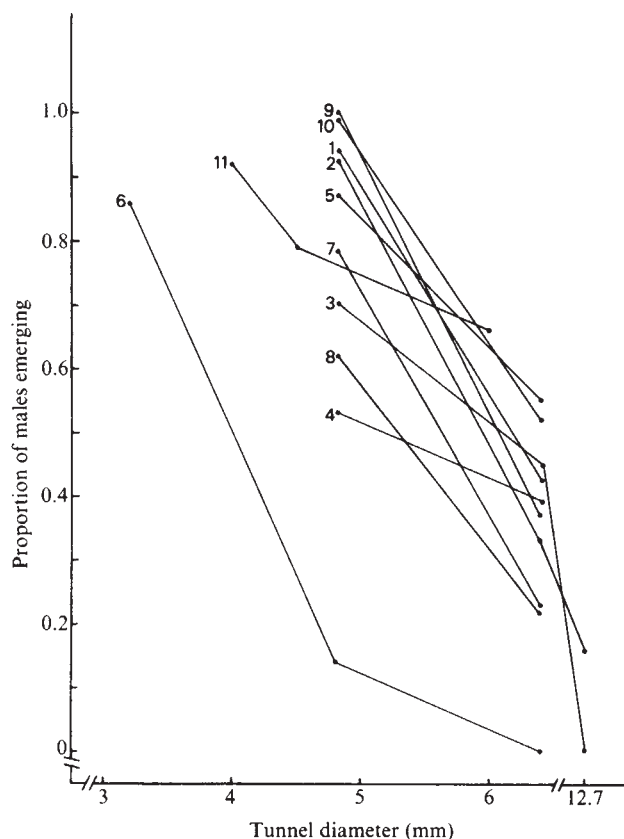


Fig. 6 Sex ratio at emergence related to tunnel diameter of nesting material for trap-nesting Hymenoptera. Data points refer to diameter of nesting material used in sampling. Lines connecting points are simply to keep species distinct. All sex ratio shifts are statistically significant at the 0.05 level (one-tailed, Fisher exact test) or better. Species 1–10 are from ref. 38, species 11 from ref. 39. Species as follows: 1, *Euodynerus foraminatus*; 2, *Euodynerus megaera*; 3, *Euodynerus schwarzi*; 4, *Pachodynerus erynnis*; 5, *Ancistrocerus antilope*; 6, *Ancistrocerus tigris*; 7, *Symmorphus canadensis*; 8, *Trypophilum tridentatum* (North Carolina); 9, *Trypophilum johannis*; 10, *Osmia lignaria*; 11, *Megachile rotundata*. Species 1–9 are wasps, 10 and 11 are bees.

Received 4 August; accepted 22 September 1980.

1. Charnov, E. L. *Am. Nat.* **113**, 465–480 (1979).
2. Maynard Smith, J. *The Evolution of Sex* (Cambridge University Press, 1978).
3. Williams, G. C. *Sex and Evolution* (Princeton University Press, 1975).
4. Charnov, E. L., Maynard Smith, J. & Bull, J. J. *Nature* **263**, 125–126 (1976).
5. Charnov, E. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2480–2484 (1979).
6. Warner, R. R. *Am. Nat.* **109**, 61–82 (1975).
7. Warner, R. R., Robertson, D. R. & Leigh, E. G. *Science* **190**, 633–638 (1975).
8. Leigh, E. G. Jr, Charnov, E. L. & Warner, R. R. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3656–3660 (1976).
9. Ghiselin, M. T. *Q. Rev. Biol.* **44**, 189–208 (1969).
10. Ghiselin, M. T. *The Economy of Nature and the Evolution of Sex* (University of California Press, 1974).
11. Hoagland, K. E. *Malacologia* **17**, 365–391 (1978).
12. Charnov, E. L. *Am. Nat.* **113**, 715–734 (1979).
13. Lloyd, D. G. N. Z. J. Bot. **17**, 595–606 (1979).
14. Charlesworth, B. & Charlesworth, D. *Am. Nat.* **112**, 975–997 (1978).
15. Willson, M. F. *Am. Nat.* **113**, 777–790 (1979).
16. Bawa, K. S. *Ann. Rev. Ecol. Syst.* **11** (in the press).
17. Maynard Smith, J. *Am. Scientist* **64**, 41–45 (1976).
18. Fisher, R. A. *The Genetical Theory of Natural Selection* (Oxford University Press, 1930).
19. Hamilton, W. D. *Science* **156**, 477–488 (1967).
20. Trivers, R. L. & Hare, H. *Science* **191**, 249–263 (1976).
21. Charnov, E. L. *Am. Nat.* **112**, 317–326 (1978).
22. Assem, J. van den Neth. J. Zool. **21**, 373–402 (1971).
23. Chweyreu, I. C. r. Séanc. Soc. Biol. **74**, 695–699 (1913).
24. Sandlan, K. *Ecol. Ent.* **4**, 365–378 (1979).
25. Clausen, C. D. *J. N. Y. ent. Soc.* **47**, 1–9 (1939).
26. Holdaway, F. T. & Smith, H. F. *Aust. J. exp. Biol. med. Sci.* **10**, 247–159 (1933).
27. Brunson, M. H. *Science* **86**, 197 (1937).
28. Nozato, K. *Kontyu* **37**, 134–146 (1969).
29. Assem, J. van den Neth. J. Zool. **20**, 329–352 (1970).
30. Assem, J. van den in *Sluipwespen in relatie tot hun gastheren* (eds Klomp, H. & Wiebes, J. T.) 64–96 (Centrum voor Landbouwpublikaties en documentatie, Wageningen, 1979).
31. Arthur, A. P. & Wylie, H. G. *Entomophaga* **4**, 297–309 (1959).
32. Ryan, R. B. & Rudinsky, J. A. *Can. Ent.* **94**, 748–763 (1962).
33. Berry, P. A. *J. Econ. Ent.* **32**, 717–721 (1939).
34. Kishi, Y. *Appl. ent. Zool.* **5**, 126–132 (1970).
35. Trivers, R. L. & Willard, D. E. *Science* **179**, 90–92 (1973).
36. Hamilton, W. D. in *Sexual Selection and Reproductive Competition in Insects* (eds Blum, M. S. & Blum, N. A.), (Academic, New York, 1979).
37. Kirkpatrick, R. L. & Wilbur, D. A. *J. econ. Ent.* **58**, 979–985 (1965).
38. Krombein, K. V. *Trap Nesting Wasps and Bees* (Smithsonian, Washington, DC, 1968).
39. Stephen, W. P. & Osgood, C. E. *J. econ. Ent.* **58**, 965–968 (1965).
40. Baroni Urbani, C. in *Social Insects* Vol. 1 (ed. Hermann, H. R.) 19–121 (Academic, New York, 1979).
41. Hamilton, W. D. *J. theor. Biol.* **7**, 1–52 (1964).
42. Charnov, E. L., Gotshall, D. & Robinson, J. *Science* **200**, 204–206 (1978).
43. Gregg, K. *Selbyana* **1**, 101–113 (1975).
44. Peterson, J. J. *J. Nematol.* **4**, 83–87 (1972).
45. Poinar, G. O. Jr *Nematodes for Biological Control of Insects* (CRC Press, Boca Raton, Florida, 1979).
46. Shapiro, D. Y. *Adv. Study Behav.* **10**, 43–102 (1979).
47. Charnov, E. L. & Bull, J. J. *Nature* **266**, 828–830 (1977).
48. Freeman, D. C., Harper, K. T. & Charnov, E. L. *Oecologia* (in the press).

Isolation of a gene from *Drosophila* by complementation in yeast

Steven Henikoff*, Kelly Tatchell†, Benjamin D. Hall† & Kim A. Nasmyth†

* Department of Zoology and † Department of Genetics, University of Washington, Seattle, Washington 98195

Transformation of mutant yeast cells by cloned genomic DNA from a higher eukaryote has made it possible to isolate a Drosophila DNA sequence that complements a yeast adenine-8 mutation. A 0.8-kilobase poly(A)-containing RNA is transcribed from the cloned Drosophila segment in transformed yeast cells and can account for functional expression of the gene.

PHYSICAL analysis of a large number of genes from animals has yielded an overall picture of the eukaryotic gene as a complex entity, sandwiched between long untranslated regions and interrupted several times by sequences which are not present in the mature mRNA. The procedures for isolating animal genes generally depend on specific nucleic acid sequence probes obtained by virtue of an enriched content of a particular mRNA in some specialized tissue or developmental stage. Consequently, the animal genes that have been most studied are seldom expressed in more than a few cell types. It is possible that some of the structural features attributed to higher eukaryotic genes are only typical of genes for differentiated functions. It is not known whether genes for cellular metabolism and cell division show these same features because of the absence of methods for isolating them from animal cells.

We now describe a gene substitution method for the isolation and molecular genetic analysis of metabolic genes from higher eukaryotes. The method is based on functional complementation of mutant yeast cells by recombinant plasmids from a random genomic library of *Drosophila* DNA fragments inserted into a yeast vector. By this procedure we have cloned a *Drosophila* gene that complements a mutation at the yeast adenine-8 locus.

Yeast transformation with pYF DNA

The cloning of yeast genes by complementation of yeast mutations has been successfully applied for genes affecting both cell metabolism^{1–3} and the cell cycle⁴. In our experiments adenine auxotrophs were chosen because all organisms are thought to synthesize purines by a universal *de novo* pathway. We constructed recombinant plasmids by inserting *Drosophila* genomic fragments into a vector that could be replicated and selected for in both *Escherichia coli* and yeast. *Drosophila melanogaster*

embryonic DNA was partially digested with *Sau3A*, a restriction endonuclease that recognizes GATC, cutting 5' to the G to leave single-stranded GATC ends⁴. A mixture of such *Sau3A* fragments with a mean size of ~5 kilobases was then ligated into the single *Bam*HI restriction site (G↓GATCC) of the plasmid YEp13 (ref. 2), and the ligation mix was used to transform *E. coli* to give a quasi-random pool of cloned *Drosophila* fragments. Figure 1 shows the structure of a pYF plasmid. The bacterial ampicillin resistance gene of pBR322 allows selection in *E. coli* and the yeast *LEU2* gene allows selection in *Saccharomyces cerevisiae*. The presence of yeast 2-μm circle DNA permits plasmid replication in yeast without integration.

A plasmid DNA pool containing many YEp13-*Drosophila* recombinants was purified from *E. coli* and used to transform mutant yeast strains^{4,7}. To determine the adequacy of selective conditions, DNA from an identically constructed yeast DNA pYe pool was used to transform aliquots of mutant cells. Cells mutant for both *leu2* and the test marker were plated in the absence of both leucine and adenine, so that only cells carrying a plasmid with both *LEU2* and the Ade⁺ gene being tested should have grown. We tested six purine auxotrophic strains, *ade1-ade4*, *ade5,7* and *ade8*. Ade⁺ prototrophs were found in every case for cells transformed with the pYe pool, except for *ade2*. (The pYe pool was constructed with DNA from an *ade2* mutant strain.) For cells transformed with the pYF pool, Ade⁺ prototrophs were found in one case out of six—*ade8*.

For *ade8*, six independent prototrophs were found from the pYF pool—these were picked up at a frequency of about 1 per 10⁴ Leu⁺ transformants whereas prototrophs from the pYe pool were picked up about an order of magnitude more frequently. Because the genome size of *Drosophila* is about 10 times that of yeast, one would expect the fly *ADE8* gene to be present ~1/10th as often in the pYF pool as its counterpart in the pYe

pool. These pYF and pYe prototrophs were shown to be genuine *ADE8* transformants rather than revertants or contaminants by the behaviour of these cells when grown without selection. YEp13 plasmids are lost at a frequency of ~1% per cell generation from yeast so that after several generations of growth on complete medium, a large fraction of cells will be leucine-requiring. Such cells should also be *ade8* if *ADE8* is carried on the recombinant plasmid. By this test, all *ADE8* transformants, whether derived from the pYe or from the pYF pool, were found to be carried on a *LEU2* plasmid—that is, loss of *Leu*⁺ was always accompanied by adenine auxotrophy.

Restriction analysis of *ADE8* plasmids

DNA was isolated from the six pYF and from six pYe *ADE8* transformants grown up with adenine selection. DNA samples were used to transform *E. coli*, from which single ampicillin-resistant clones were grown and plasmid DNA extracted. Restriction endonuclease analysis of these plasmids is sum-

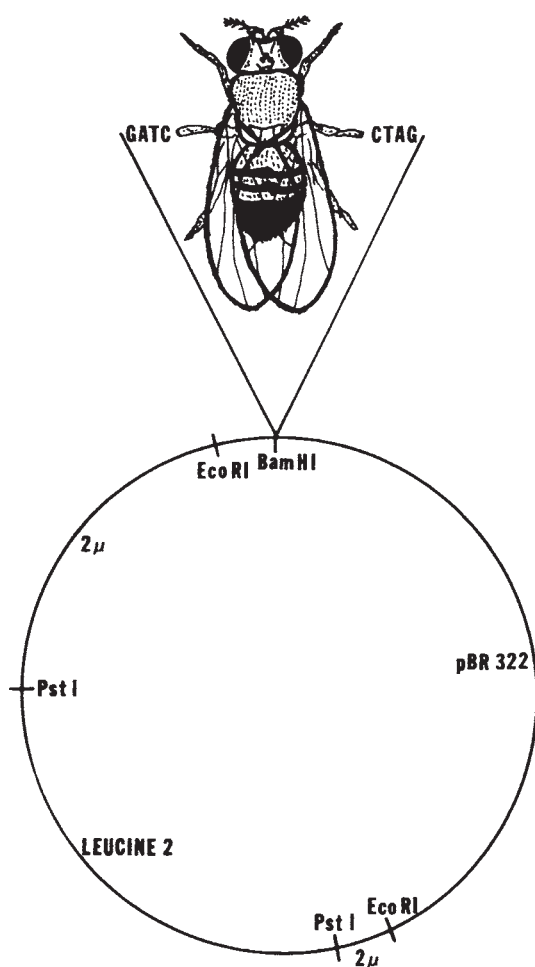


Fig. 1 Schematic map of pYF plasmid DNA derived from the bacterial plasmid pBR322, yeast 2-μm circle DNA, the yeast *LEU2* gene and *Drosophila* embryonic DNA. Fresh *D. melanogaster* (wild-type 'Sevelin') embryos were thoroughly washed and dechorionated 8–15 h after egg deposition, and 2,000g nuclei were prepared⁵. Microbial contamination was checked by plating out and was found to be insignificant. Nuclei were lysed in 10 mM Tris pH 7.9, 4 mM EDTA, 1% Sarkosyl, and DNA was banded in CsCl and dialysed. Partial digestion of this DNA with *Sau3A*, ligation into YEp13 (ref. 2) and transformation was performed as described elsewhere⁴. A pool of *E. coli* transformants (3×10^5) with mean *Drosophila* insert size of 4–5 kilobases was obtained and was enriched by selection for ampicillin resistance and tetracycline sensitivity⁶. A log-phase culture inhibited with chloramphenicol was used for plasmid DNA isolation and purification⁴.

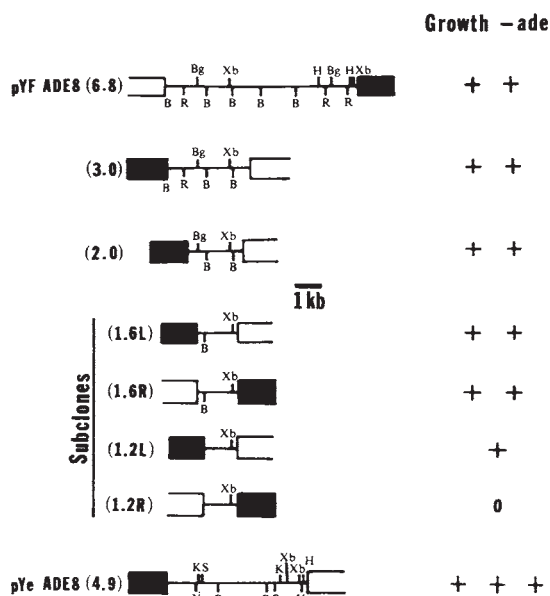


Fig. 2 Restriction endonuclease analysis of *ADE8* plasmid inserts and relative growth with adenine selection of *ade8* cells carrying *ADE8* plasmids. Orientation of the inserts within the YEp13 vector is indicated by the solid panel, which corresponds to the region on the left hand side of the *Drosophila* segment in Fig. 1. Subclones were constructed by ligating the gel-purified 1.6-kilobase *Bgl*II–*Bam*HI and the 1.2-kilobase *Bam*HI–*Bam*HI fragments, derived from pYF *ADE8*(3.0) and pYF *ADE8*(2.0) respectively, into the *Bam*HI site of YEp13 (ref. 2). All pYF *ADE8* inserts appeared to overlap with one another but showed no similarity to the pYe *ADE8* inserts, one example of which is shown. The following enzymes were used: *Bam*HI (B), *Bgl*II (Bg), *Hind*III (H), *Kpn*I (K), *Eco*RI (R), *Sal*I (S), *Xba*I (Xb), and *Xho*I (X). Growth rates were determined by colony size estimation for initial isolates and subclones tested in the same yeast strain used for the initial selection (5028-1Dα *ade8*-18 *leu2*-1 *met2* *his4*-4 *lysX* gal cup⁷). Transformants were tested on plates minus leucine and on plates minus adenine. No differences in colony size were noted for any transformant using leucine selection. Growth using adenine selection was estimated from colony size after 3 days at 29 °C. kb, Kilobase.

marized in Fig. 2. The six pYF plasmids showed three different but partially overlapping restriction patterns, and the six pYe plasmids showed two. Apparently the smallest pYF insert is contained entirely within the other two: it has the same orientation as the second longest. This was confirmed by hybridization analysis using one of the plasmids as a probe (data not shown). No cross homology was detected, however, between the pYF and the pYe *ADE8* plasmid inserts.

To demonstrate directly the presence of an *ADE8* gene, *ade8 leu2* yeast cells were transformed with each one of the plasmids being analysed. In every case, both *ade8* and *leu2* were simultaneously complemented, proving that an *ADE8* gene is present within each plasmid insert (data not shown). Using leucine selection, all such transformants grow about as well as wild-type cells regardless of which plasmid is used. However, when adenine is absent, an interesting difference appears. Whereas yeast cells carrying pYe-derived plasmids grow about as rapidly as do wild type, those with any of the pYF plasmids grow at about two-thirds the normal rate. This reduced rate of growth attributable to the pYF *ADE8* gene suggests that the *Drosophila ADE8* gene functions less effectively in yeast than its yeast functional counterpart.

Hybridization of pYF *ADE8* to *Drosophila* DNA

An unlikely artefact that could account for complementation of a yeast auxotroph by pYF plasmids is microbial contamination of the *Drosophila* DNA, although precautions were taken to prepare very clean dechorionated embryos. Southern transfer

experiments can rule out this possibility by demonstrating that the pYF plasmid inserts are derived from *Drosophila*⁸. Figure 3 shows an agarose gel on which *Drosophila* genomic DNA was electrophoresed after cutting with *Bam*HI restriction enzyme. In another lane, an amount of pYF ADE8(2.0) representing a genome equivalent was also digested with *Bam*HI and electrophoresed. This gel was blotted and probed with the 1,200-base pair fragment produced by *Bam*HI digestion of pYF ADE8(2.0) and labelled with ³²P by nick-translation. Figure 3c, d shows that a 1,200-base pair band is present in both lanes which indicates that the pYF ADE8 inserts are derived from *Drosophila*. Also, the extent of hybridization is the same in both lanes which suggests that the sequence is single-copy.

This result has been confirmed using *Drosophila* genomic DNA from embryos of different strains and from polytene fat body cut with *Eco*RI, *Xba*I and *Bgl*II. In each case, a single band of the expected size was hybridized to the extent expected for a sequence present once per haploid genome (data not shown). Thus we conclude that the pYF sequence complementing *ade8* is derived from *Drosophila* and is single-copy.

A *Drosophila* sequence present once per haploid genome in polytene tissue can be localized unambiguously on salivary chromosomes. Figure 4 shows the result of an *in situ* hybridization experiment using ³H cRNA to pYF ADE8(6.8) as a probe. A single site, 27C on chromosome arm 2L, is labelled. Even exposures 10 times longer than for the example in Fig. 4 do not show labelling of any other site. The region around the labelled site is not one of extensive genetic characterization¹³. However, in screening for adenosine auxotrophs on chromosome 2, Naguib and Nash (personal communication) have isolated two non-allelic *Drosophila* mutations, and both map to this region. It is tempting to speculate that one of these auxotrophic mutations blocks the reaction catalysed by the *Drosophila* enzyme that is coded for by pYF ADE8. In yeast, adenine-8 is presumed to be the gene for the enzyme glycylamide ribotide (GAR) trans-formylase¹⁴. It will be interesting to determine whether the same enzyme activity is specified by this sequence in *Drosophila*.

Transcription of pYF ADE8

Because the *Drosophila* segments that complement *ade8* were carried on plasmids that also included bacterial and yeast DNA, we wondered whether or not ADE8 gene expression depends entirely on *Drosophila* DNA. For example, transcription and translation could conceivably initiate or terminate within flanking vector DNA so that the *Drosophila* sequence would supply only structural information. We found that this was not likely.

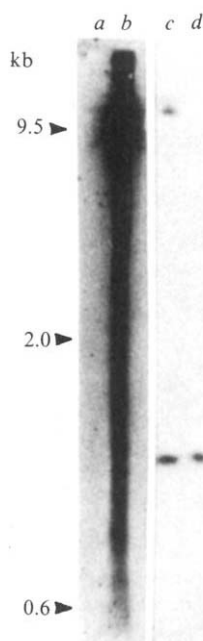


Fig. 3 Hybridization of *Drosophila* genomic DNA with pYF ADE8 probe. a, An aliquot (3 ng) of pYF ADE8(2.0) DNA was digested with *Bam*HI and electrophoresed on a 0.9% agarose gel⁹ adjacent to b: 10 µg *Bam*HI-digested *Drosophila* embryonic DNA. After staining with ethidium bromide and photographing, the DNA was transferred to nitrocellulose and hybridized¹⁰ with the 1,200-base pair *Bam*HI fragment partially purified (95%) from pYF ADE8(2.0) and labelled with ³²P by nick-translation. The nick-translation reaction mixture (50 µl) contained 10 µCi [α -³²P]dCTP (NEN); 4 µM dATP, dGTP and dTTP; 50 mM Tris-HCl pH 8; 5 mM MgCl₂; 50 µg per ml bovine serum albumin; ~1 µg DNA; 3 units DNA polymerase I (Boehringer) and 75 pg DNase I (Worthington). c, d, Autoradiograms of tracks a and b, respectively.

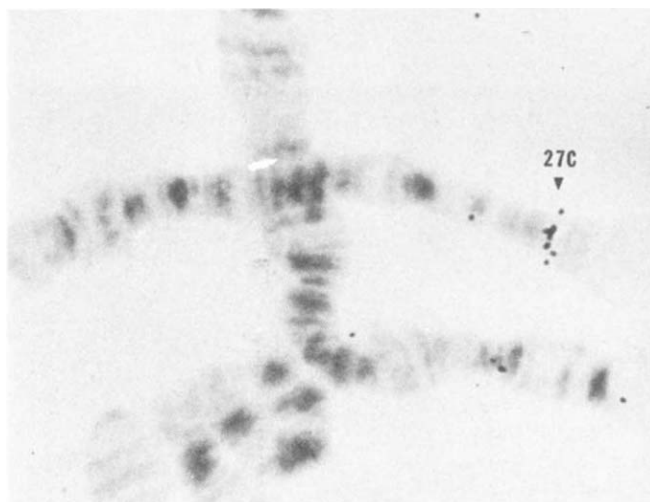


Fig. 4 Hybridization of pYF ADE8(6.8) to polytene chromosomes *in situ*. ³H-labelled copy RNA¹¹ was hybridized as described elsewhere¹² to *gt w^a/gt^{X11}* prepupae. Exposure was for 3 days.

The gene functioned at the same reduced rate for all three different plasmid types, even though their inserts had different orientations. More direct evidence that ADE8 gene expression depended on *Drosophila* DNA and not flanking vector sequences came from an investigation of transcription on a pYF ADE8 plasmid.

If pYF ADE8 complements the yeast *ade8* mutation by making a protein, then one would expect to find poly(A)-containing RNA hybridizable to *Drosophila* ADE8 DNA in cells transformed with pYF ADE8 plasmids. Adenine-8 mutant cells that carry pYF ADE8(2.0) were grown with adenine selection and poly(A)⁺ RNA was isolated. An aliquot of this RNA was denatured in dimethyl sulphoxide (DMSO) and glyoxylated, then electrophoresed on agarose and transferred to nitrocellulose as described elsewhere¹⁵. This 'Northern' transfer was probed with the 1.5-kilobase fragment derived from the pYF ADE8(2.0) insert by digestion with *Bgl*II and *Xba*I and labelled with ³²P by nick-translation. Figure 5 shows the resulting autoradiogram. Two prominent bands are seen, one at 0.8 kilobases and another at 2.2 kilobases, with a few minor bands and some heterogeneous labelling also visible. The 2.2-kilobase transcript is very likely to have been read from vector sequences in part, as it seems to be longer than the entire 2-kilobase *Drosophila* insert in the plasmid that was carried by the cells from which the RNA was derived. Experiments described below demonstrate that the 0.8-kilobase transcript is derived entirely from *Drosophila* DNA, and that it is responsible for ADE8 function.

One way to determine which transcript provides ADE8 function is physically to map the transcript on the plasmid, then compare this map with a functional map determined by testing partially deleted subclones for *in vivo* function. We used S₁ nuclease protection of transcripts by various fragments to map the transcribed region¹⁸. Poly(A)⁺ RNA used in the above Northern blot experiment was hybridized to the 1,500-base pair *Bgl*II-*Xba*I fragment in conditions that allow RNA-DNA but not DNA-DNA duplex formation. In parallel reaction mixtures, the DNA fragment was also first digested with either *Bam*HI or *Ava*II to give fragments that were 350 or 375 base pairs shorter, as shown in Fig. 6a. These mixtures were then digested with S₁ nuclease, electrophoresed on an agarose gel and transferred to nitrocellulose. This blot was probed with pYF ADE8(2.0) labelled with ³²P by nick-translation and autoradiographed—the result is shown in Fig. 6a. All three fragments are protected by transcript to give a major band at ~0.8 kilobases and a minor band at ~0.7 kilobases. Both these bands are the same size when either the *Bgl*II-*Xba*I fragment or

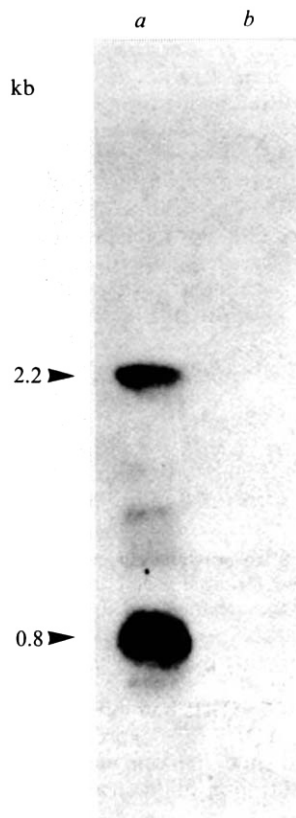


Fig. 5 Hybridization of pYF *ADE8* DNA to a Northern blot of poly(A)-containing RNA. Log-phase *ade8 leu2* yeast cells carrying *a*, pYF *ADE8*(2.0) and *b*, pYE *ADE8*(4.9) were grown with adenine selection. RNA was extracted¹⁶ and the poly(A)⁺ fraction isolated¹⁷. Samples (10 µg) were glyoxylated in 80% DMSO at 65 °C, electrophoresed, transferred to nitrocellulose and hybridized using the procedure of Thomas¹⁵. The probe used was the purified *Bgl*II-*Xba*I fragment derived from the insert of pYF *ADE8*(3.0) purified on a 1.1% agarose gel⁹, electroeluted and labelled with ³²P by nick-translation.

the *Bam*HI-*Xba*I fragment is used for hybridization but are reduced in size by about 25 nucleotides when the *Ava*II-*Xba*I fragment is used. This result allows assignment of both transcripts to a region stretching from the *Bam*HI site to a point 0.8 kilobases to the right for the major transcript and 0.7 kilobases for the minor. This minor transcript is probably produced as a result of the S₁ digestion procedure because in milder conditions it is absent (data not shown). An S₁ nuclease-protected region of 0.8 kilobases corresponds well to the 0.8-kilobase transcript detected on the Northern blot. Failure to detect the 2.2-kilobase transcript here suggests that it is complementary to only a small part at one end of the *Bgl*II-*Xba*I fragment, and due to its small size, escapes detection. This experiment also suggests that there are no intervening sequences because the protected fragments are all of the same size in this neutral gel, as they were in an alkaline denaturing gel run in parallel (not shown).

The orientation of the 0.8-kilobase transcript was determined in a similar S₁ nuclease protection experiment using fragments labelled at one end only. Two different fragments were used (Fig. 6b). Starting with the 1.2-kilobase fragment liberated by *Bam*HI digestion, one labelled fragment (A) was made by cutting with *Ava*II, labelling the 3' ends and then cutting with *Xba*I. The other labelled fragment (B) was made similarly but without the *Ava*II digestion. Ignoring the small fragments produced, fragment A is labelled at the *Ava*II site on its 3' end and fragment B is labelled at the leftmost *Bam*HI site on its 3' end. Only if the transcript is oriented with its 5' end on the left and covers the labelled 3' end restriction site will that site be protected from S₁ nuclease digestion. This is the case for the *Ava*II site as seen in the autoradiogram of a polyacrylamide gel (Fig. 6b). For fragment B, S₁ nuclease protection is also found, although not as much as for fragment A. Partial protection of the *Bam*HI site shows that the 5' end of the 0.8-kilobase transcript is within a few nucleotides of this site.

Behaviour of subcloned pYF *ADE8* fragments *in vivo*

Our evidence that the 0.8-kilobase transcript made in yeast starts and stops within the *Drosophila* sequences of pYF *ADE8*(2.0) does not unequivocally demonstrate that this

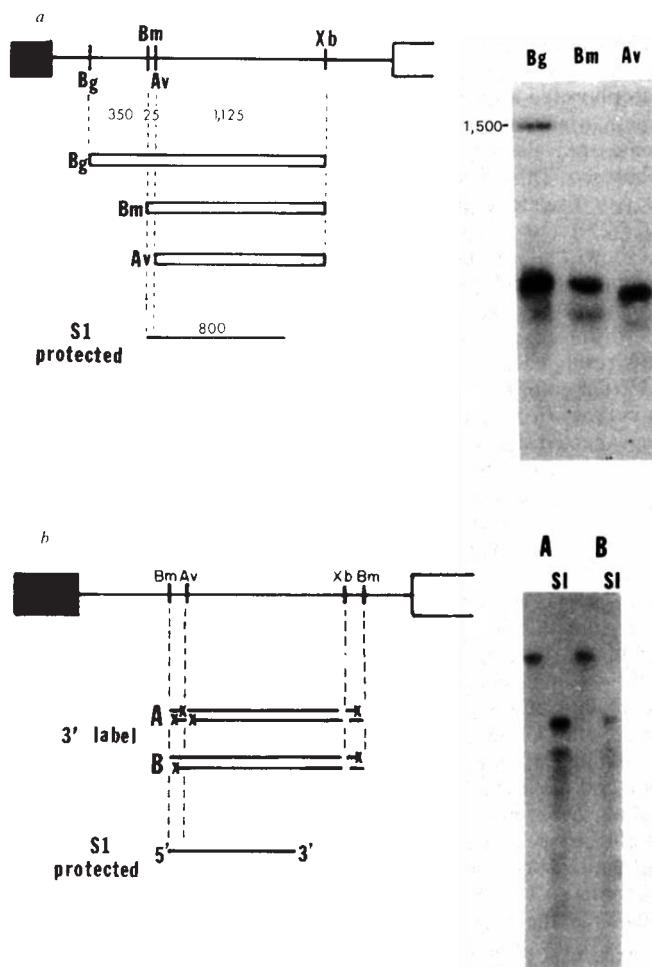


Fig. 6 Mapping of transcription by S₁ nuclease protection of restriction fragments derived from pYF *ADE8*(2.0). Purified restriction fragments were hybridized to poly(A)-containing RNA from cells carrying pYF *ADE8*(2.0) and S₁ nuclease-digested according to Holmgren *et al.*¹⁹. Each hybridization mixture (20 µl) contained 80% formamide, 0.3 M NaCl, 1 mM EDTA, 10 mM PIPES pH 6.8, 10–40 µg RNA and 80–160 ng DNA. Samples were kept at 70 °C for 10 min then transferred to 53 °C for overnight incubation. These were mixed with 20-µl aliquots of 0.3 M NaCl, 6 mM ZnCl₂, 100 mM Na-acetate, pH 4.0, and 50,000 units per ml S₁ nuclease, incubated for 5 min at 37 °C, extracted with phenol and ethanol-precipitated. *a*, Protection of unlabelled fragments. Hybrids were electrophoresed on a neutral 1.5% agarose gel, blotted onto nitrocellulose, hybridized with pYF *ADE8*(2.0) which was labelled with ³²P by nick-translation, and autoradiographed. The band at 1,500 base pairs is apparently due to DNA-DNA reassociation of the *Bgl*II-*Xba*I fragment. *b*, Protection of 3' end-labelled DNA fragments. Before hybridization, the 1.2-kilobase *Bam*HI-*Bam*HI fragment purified from pYF *ADE8*(2.0) was either digested with *Ava*II (A) or used uncut (B) to fill the 3' ends (indicated by x) with ³²P-labelled deoxynucleotide triphosphates. Both samples were digested with *Xba*I and aliquots used for hybridization and S₁ nuclease digestion. Hybrids were then redissolved in 0.1 M NaOH and electrophoresed on 4% acrylamide gels containing 7 M urea²⁰. The resulting autoradiogram is shown at the lower right. For each labelled fragment A and B, an aliquot of the fragment itself was run to the left of the S₁ nuclease-digested sample (S1). Because the specific activities of the A and B large fragments differed, their concentrations were adjusted for the gel to give the same amount of radioactivity in both untreated large fragments. For the S₁ nuclease-treated samples, the same relative adjustment in concentration was made so that these gel tracks could be quantitatively compared for A and B. The 0.7-kilobase minor band seen after S₁ nuclease digestion is probably a digestion artefact (see text). Restriction sites are *Bgl*II (Bg), *Bam*HI (Bm), *Ava*II (Av) and *Xba*I (Xb). The orientation of the poly(A)⁺ transcript is shown.

sequence is responsible for *ade8* complementation. For example, it is possible that a less abundant transcript accounts for *ADE8* function. However, the following *in vivo* tests of subcloned fragments strongly suggest that the 0.8-kilobase transcript mapped above is the functional message. As shown in Fig. 2, we tested fragments subcloned into the *Bam*HI site of YEp13 in both orientations. The 1.6-kilobase fragment made by *Bgl*II digestion and *Bam*HI partial digestion of pYF *ADE8*(2.0) showed *ADE8* complementation activity equivalent to that of the original plasmids when subcloned in either orientation. The 1.2-kilobase fragment made by complete *Bam*HI digestion of pYF *ADE8*(2.0) showed reduced *ADE8* activity in one orientation and no activity at all when subcloned in the other orientation. Because the 350 base pairs between the *Bgl*II site and the leftmost *Bam*HI site are the only difference between the subclones in either orientation, the structural gene apparently lies between the two *Bam*HI sites with a control region just to the left. This mapping of *Drosophila* adenine-8 based on function agrees well with the transcriptional mapping, because the entire transcript, except for possibly a few nucleotides at the 5' end, is read from a region that can function in a subclone. It seems likely that substitution of sequences just upstream of the structural gene, perhaps even replacing the initiation site, has reduced or eliminated gene expression. Therefore, we conclude that the 0.8-kilobase poly(A)⁺ transcript is the message in yeast and that the *Drosophila* segment from which it is transcribed is entirely responsible for complementation of *ade8*.

That this transcript functions as message is also apparent from a preliminary analysis of its nucleotide sequence. An open reading frame has been found to cover most of the transcribed region (C. E. Furlong and S.H., unpublished results). Presumably, this sequence codes for the enzyme GAR transformylase from *D. melanogaster*.

Concluding remarks

We have shown that yeast transformation can be used to isolate a gene from a higher eukaryote and to determine that transcription of the gene corresponds to its functional expression. Of the six adenine pathway genes investigated, only *Drosophila* adenine-8⁺ was isolated in yeast by our procedure. Several explanations can be offered for the failure to find complementation for mutations in the other five yeast genes, including cases

in which multi-enzyme genes in yeast may not be linked in *Drosophila* (for example, *ade3* and *ade5,7*), transcripts may not be correctly processed or efficiently translated in yeast, or enzyme products may not be sufficiently functional or stable. Our characterization of *Drosophila* adenine-8⁺ shows that it is transcribed in yeast to make a product that can substitute for the yeast *ADE8* gene product. This suggests that the requirements for functional expression of this gene are very much the same in insects and fungi. As this is one of the first higher eukaryotic genes of a general metabolic nature to be analysed, it is possible that such genes will generally be similar to those in yeast. The gene substitution method used may prove useful for further inter-kingdom comparisons.

We thank Charles Laird for support and encouragement, Carol Sandstrom for help with embryo collection, Jim Sloan for testing yeast strains, Pat Thomas for providing the RNA blotting protocol and Seymour Fogel for the *ade8* strain. This work was supported by grants from NIH to B. D. H. and to C. Laird, by a Damon Runyon-Walter Wichell Cancer Fund postdoctoral fellowship to K. T. and by a Jane Coffin Childs Memorial Fund for Medical Research postdoctoral fellowship to K. A. N.

Received 21 August; accepted 29 October 1980.

1. Hinnen, A., Farabaugh, P., Ilgen, C., Fink, G. R. & Friesen, J. *ICN-UCLA Symp. molec. cell. Biol.* **14**, 43-50 (1979).
2. Broach, J. R., Strathern, J. N. & Hicks, J. B. *Gene* **8**, 121-133 (1979).
3. Williamson, V. M., Bennetsen, J., Young, E. T., Nasmyth, K. & Hall, B. D. *Nature* **283**, 214-216 (1980).
4. Nasmyth, K. A. & Reed, S. I. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2119-2123 (1980).
5. Elgin, S. C. R. & Miller, D. W. in *The Genetics and Biology of Drosophila* Vol. 2a (eds Ashburner, M. E. & Wright, T. R. F.) 145-150 (Academic, New York, 1978).
6. Bolivar, F. *et al. Gene* **2**, 95-113 (1977).
7. Beggs, J. D. *Nature* **275**, 104-108 (1978).
8. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
9. Helling, R. B., Goodman, H. M. & Boyer, H. W. *J. Virol.* **14**, 1235-1244 (1974).
10. Wahl, G. M., Stern, M. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3683-3687 (1979).
11. Wensink, P. C., Finnegan, D. J., Donelson, J. E. & Hogness, D. S. *Cell* **3**, 315-325 (1974).
12. Henikoff, S. & Meselson, M. *Cell* **12**, 441-451 (1977).
13. Lindsley, D. L. & Grell, E. H. *Genetic Variations of Drosophila melanogaster* Publ. No. 627 (Carnegie Institution of Washington, 1968).
14. Woods, R. & Jackson, I. *Biochem. biophys. Res. Commun.* **53**, 787-793 (1973).
15. Thomas, P. *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201-5205 (1980).
16. Schultz, L. D. *Biochemistry* **17**, 750-758 (1978).
17. Aviv, H. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **69**, 1408-1412 (1972).
18. Berk, A. J. & Sharp, P. A. *Cell* **12**, 721-732.
19. Holmgren, R., Livak, K., Morimoto, R., Freund, R. & Meselson, M. *Cell* **18**, 1359-1370 (1979).
20. Maxam, A. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-564 (1977).

Intervening sequences in a plant gene—comparison of the partial sequence of cDNA and genomic DNA of French bean phaseolin

S. M. Sun[†], J. L. Slightom^{*†} & T. C. Hall^{||}

Departments of Horticulture and Genetics*, University of Wisconsin, Madison, Wisconsin 53706

A plant gene coding for the major storage protein (phaseolin, G1-globulin) of the French bean was isolated from a genomic library constructed in the phage vector Charon 24A. Comparison of the nucleotide sequence of part of the gene with that of the cloned messenger RNA (cDNA) revealed the presence of three intervening sequences, all beginning with GT and ending with AG. The 5' and 3' boundaries of intervening sequences IVS-A (88 base pairs) and IVS-B (124 base pairs) are similar to those described for animal and viral genes, but the 3' boundary of IVS-C (129 base pairs) shows some differences. A sequence of 185 amino acids deduced from the cloned DNAs represents about 40% of a phaseolin polypeptide.

PHASEOLIN, the first globulin (G1) fraction to precipitate on dilution of an acidic saline extract¹, is the major storage protein

[†] Present addresses: Atlantic Richfield Co., 6905 Sierra Court, PO Box 2600, Dublin, California 94566 (S.M.S.); Departments of Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois 62901 (J.L.S.).

^{||} To whom correspondence should be addressed.

in the seeds of French bean (*Phaseolus vulgaris* L.). One-dimensional SDS-polyacrylamide gel electrophoresis of phaseolin from cotyledons of the cultivar 'Tendergreen' resolves three polypeptides, α , β , and γ , with apparent molecular weights of 53,000, 47,000 and 43,000, respectively². Analytical ultracentrifugation reveals the free polypeptide (3S) conformation in highly alkaline (>pH 11) solutions (or in solution containing SDS), but as the pH values are lowered (in the

absence of dissociating agents), reversible association to 7.1S protomeric (at neutral pH values) and 18.2S tetrameric (at pH 3.5) forms occurs³⁻⁵. Mapping according to the method of Cleveland *et al.*⁶ established that the α , β and γ polypeptides share a general sequence homology for some 60% of their length⁷. However, two-dimensional electrophoresis⁸ shows the presence of charge microheterogeneity⁹. It is likely, therefore, that phaseolin is encoded by a multigene family, of an as yet undetermined size. Similar, or even more complex, situations are indicated for other seed crop proteins such as zein¹⁰⁻¹² from corn and the soybean storage proteins^{13,14}.

Phaseolin accumulates rapidly in the developing seed cotyledon over a 2- to 3-week period following anthesis^{15,16}. Indeed, we estimated that, at certain stages of development, phaseolin polypeptides represent some 90% of the total protein synthetic activity of this tissue. At maturity it comprises about 50% of the total protein stored in the seed¹⁷. Phaseolin does not occur anywhere in the bean other than in the cotyledon¹⁵; hence, its synthesis is under strict tissue and temporal regulation.

We describe here the isolation and characterization of a phaseolin gene. This is a major step towards investigating the expression of this economically important protein at the molecular level, and subsequently, in the use of molecular biological techniques to improve the bean as a crop plant¹⁸.

Preparation of bean DNA and construction of a genomic library

The preparation of a genomic library¹⁹⁻²¹ of bean DNA was particularly attractive because it makes possible the isolation of phaseolin and other genes of specific interest so that their structure, organization and regulation can be studied. It also facilitates determination of the number of genes coding for phaseolin polypeptides, their variability and cellular location. Only limited information concerning these questions can be obtained by classical genetic techniques. An initial step in

preparing such a library was the isolation of DNA of adequate length, free from contaminating polysaccharides or RNA, yet without nicks. We could prepare DNA of over 60 kilobase pairs in length from embryonic axes dissected from dry bean seeds (see Fig. 1 legend and ref. 22). After partial digestion of the DNA with *Eco*RI endonuclease, DNA fragments of 8–20 kilobase pairs were selected²³ and inserted into the phage λ cloning vector, Charon 24A. Approximately 2×10^6 recombinant phages were screened for phaseolin sequences using a ³²P-cDNA probe made from highly purified phaseolin mRNA^{23,24}. From 12 candidate clones, two, Ch24A-176.2 and Ch24A-177.4, were selected for further characterization.

Characterization of cloned DNA

Restriction analysis of 'mini-lysates'¹⁹ of clones Ch24A-176.2 and Ch24A-177.4 showed that they both contained a 7.2-kilobase *Eco*RI fragment (Fig. 1, lanes b, c) that hybridized strongly to the cDNA probe (Fig. 1, lanes d, e). DNA from clone Ch24A-177.4 was purified²⁵ for further analysis. This revealed (Fig. 1, lanes f, g) that, in addition to a strongly hybridizing 7.2-kilobase fragment, weak hybridization to a 1.7-kilobase fragment could also be discerned. An additional 2.2-kilobase fragment was detected that showed no hybridization to the probe. To facilitate the preparation of large quantities of cloned DNA, the 7.2- and 1.7-kilobase DNA fragments were subcloned into pBR322 (refs 20, 26).

Although the material used as a template for the synthesis of the ³²P-cDNA probe was known to be highly enriched in phaseolin mRNA^{23,27}, it was necessary to confirm that the cloned DNA did contain a phaseolin gene sequence. The first confirmation was obtained by electrophoretic separation of bean 18S and 28S rRNAs, poly(A)⁺-RNA, total poly(A)⁺-RNA, and the 16S fraction of poly(A)⁺-RNA (phaseolin mRNA) in an agarose gel (Fig. 2A). After electrophoresis, the RNAs were transferred to diazobenzyloxymethyl (DBM)-paper^{28,29} and then hybridized to the 7.2-kilobase cloned DNA fragment which was ³²P-labelled by nick-translation³⁰. No hybridization to rRNA

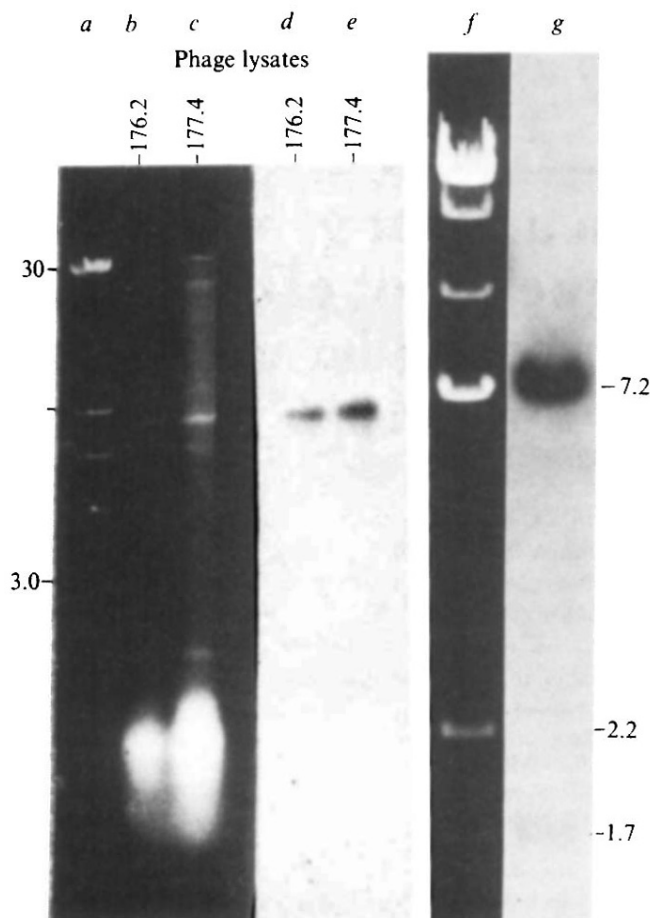
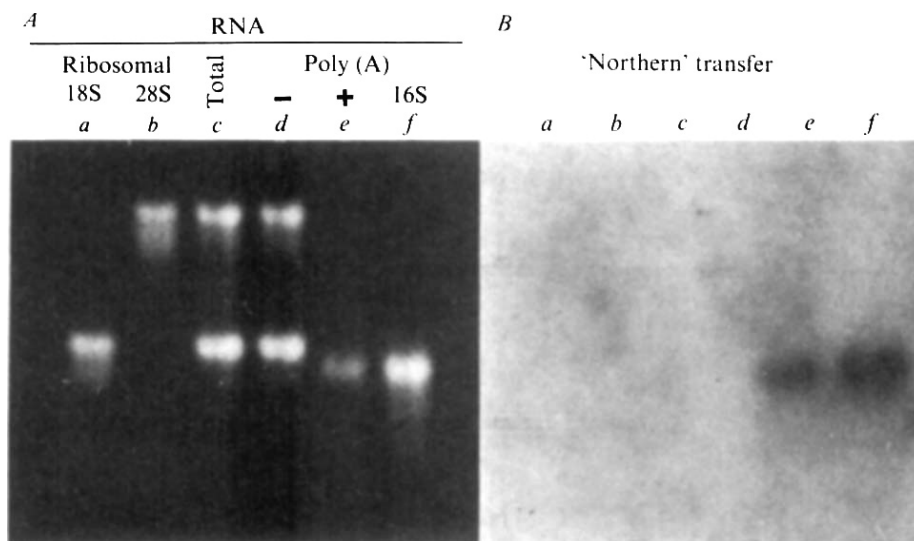


Fig. 1 Identification and selection of clones containing phaseolin sequences from a bean DNA library. French bean genomic DNA was isolated from embryo axes of dry seeds by the method of Blin and Stafford²² with the following modifications. After extraction with phenol and dialysis, the DNA solution was adjusted to 0.95 g ml⁻¹ CsCl and 0.5 mg ml⁻¹ ethidium bromide, then centrifuged twice for 60 h (45,000 r.p.m., Beckman Ti-60) at 20 °C to band the DNA, which was recovered from the gradient after each cycle. Ethidium bromide was removed by several extractions with CsCl-saturated isopropyl alcohol and the DNA dialysed against 10 mM Tris-Cl, pH 8.0, 5 mM NaCl and 1 mM EDTA before use. The DNA obtained had a length of about 60 kilobase pairs. An additional extraction with phenol was necessary after the dialysis step to obtain normal *Eco*RI digestion kinetics and higher cloning efficiencies. The construction of a 'shotgun' collection of 1.5×10^6 Charon 24A bacteriophage recombinants containing partial *Eco*RI fragments from 10 μ g of genomic DNA was as described by Blattner *et al.*¹⁹ and Slightom *et al.*²⁰. This original library was amplified to 2×10^9 recombinant phages and approximately 2×10^6 of these were screened¹⁹ using a ³²P-labelled phaseolin cDNA probe prepared according to Kacian and Myers²⁴. DNA was extracted from mini-lysates¹⁹ of two clones that showed especially strong plaque hybridization to the probe. *Eco*RI digests of these clones were electrophoresed in a 0.7% agarose gel (lane b, clone 176.2; lane c, clone 177.4; lane a contained marker DNAs¹⁹ whose sizes are shown at the left in kilobases). The DNA was then transferred to nitrocellulose paper by the method of Southern⁴⁰ and hybridized to the phaseolin ³²P-cDNA probe. The 7.2-kilobase *Eco*RI fragment from each clone hybridized strongly to the probe (lanes d, e). After purification of DNA from clone Ch24A-177.4 and digestion with *Eco*RI, two additional bands (2.2 and 1.7 kilobases, see lane f) were revealed. The ³²P-cDNA probe hybridized weakly to the 1.7-kilobase fragment but showed no recognition of the 2.2-kilobase fragment (lane g).

Fig. 2 Hybridization of cloned DNA to bean RNAs. Total RNA was isolated from maturing French bean cotyledons (grown in the Madison campus Biotron) using a hot ($\sim 70^\circ\text{C}$) Na borate-NaOH (0.2 M, pH 9.0) extraction buffer containing 1% SDS, 30 mM EGTA and 5 mM dithiothreitol, followed by deproteinization at 37°C with proteinase K (Beckman) as described by Buchbinder⁴¹ and Hall *et al.*^{23,27}. The total RNA was separated into poly(A)⁺ and poly(A)⁻ fractions by oligo(dT)-cellulose chromatography. The poly(A)⁺RNA was fractionated by centrifugation in a linear-log⁴² sucrose gradient (0–32.5% sucrose in 0.1 M NaOAc, 0.1 M NaCl and 1 mM EDTA, pH 5.0), and the 16S fraction collected. These RNA fractions, together with 18S and 28S bean rRNAs, were electrophoretically separated on a 1.5% agarose gel containing 6% formaldehyde and stained with ethidium bromide (A: lane a, 18S rRNA; lane b, 28S rRNA; lane c, total RNA; lane d, poly(A)⁻ RNA; lane e, poly(A)⁺ RNA; lane f, 16S RNA), and subsequently transferred to DBM-paper (Schleicher and Schuell) according to the procedures of Alwine *et al.*²⁸ and Rave *et al.*²⁹. The RNAs bound to the paper were then hybridized to the cloned 7.2-kilobase bean DNA fragment labelled with ³²P by nick translation³⁰. As seen in B, the radioactive cloned DNA hybridized to only the poly(A)⁺ (lane e) and 16S (lane f) RNAs.

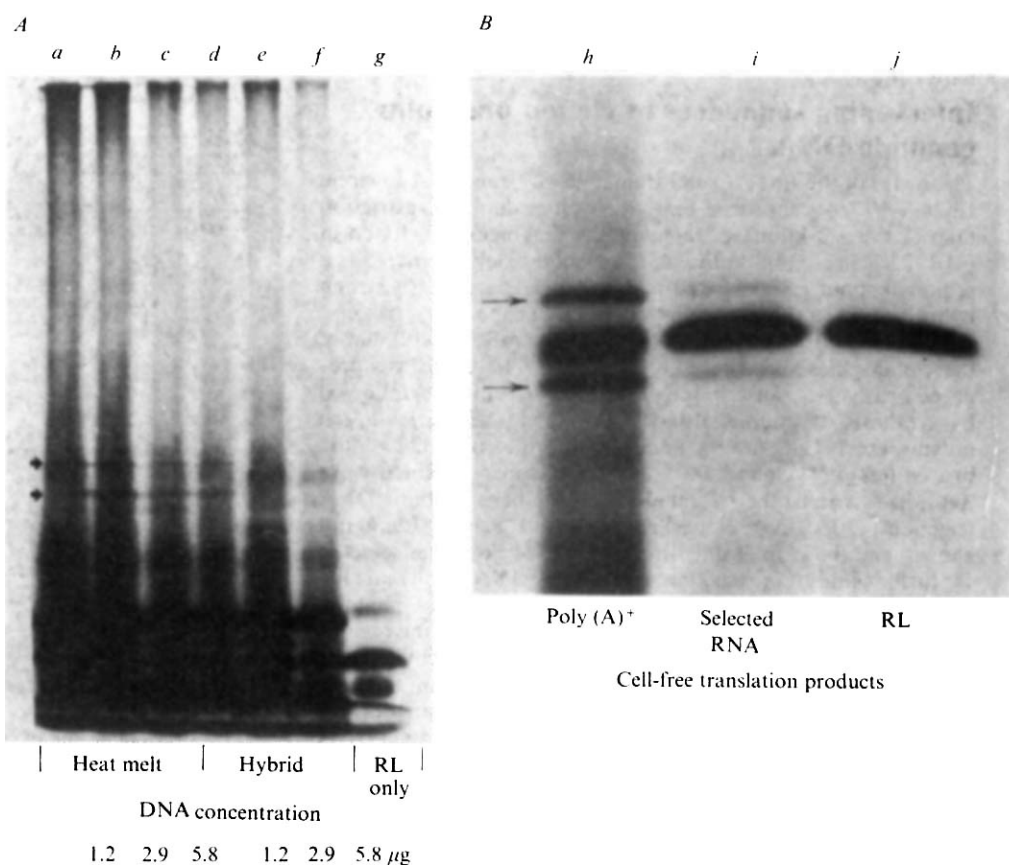


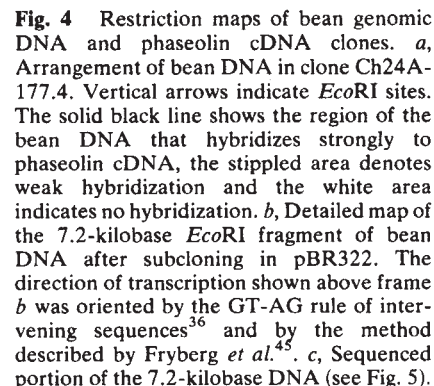
was seen, only the total poly(A)⁺ and the phaseolin mRNAs showed positive reactions (Fig. 2B). This established that the clone does not contain any rRNA sequences and shows that it reacts with the expected poly(A)⁺RNA fractions.

A second confirmation came from hybrid-arrested translation³¹, in which only those mRNAs complementary to the supplied DNA sequence are prevented from functioning as

messengers during cell-free protein synthesis. Figure 3A shows that increasing amounts of cloned DNA competed out products identified as phaseolin polypeptides, although synthesis of the other products coded by the bean cotyledon poly(A)⁺RNA was unaffected. Further confirmation that a phaseolin gene had been isolated was obtained by binding the recombinant DNA to a nitrocellulose filter and hybrid-selecting³² phaseolin mRNA

Fig. 3 Hybrid-arrested and hybrid-selected translation of phaseolin mRNA. A, Hybrid-arrested translation³¹ in the rabbit reticulocyte system was performed using various amounts (2.4 μg , 5.8 μg or 11.6 μg) of *Eco*RI-digested Ch24A-177.4 DNA hybridized to 0.4 μg bean poly(A)⁺RNA for 2 h at 48°C in the presence of 80% deionized formamide. The samples (250 μl) were each divided into two equal portions, one of which was heat melted at 100°C for 60 s before translation (lanes a, b, c; heat melt) and the other allowed to remain in hybrid form during translation (lanes d, e, f; hybrid). A reaction containing no added mRNA is shown for comparison in lane g (RL only). The ³⁵S-Met-labelled products were separated on a 13% SDS-polyacrylamide slab gel⁴³ and subjected to fluorography⁴⁴. The arrows indicate the position of phaseolin polypeptides. B, Phaseolin mRNA was selected from total poly(A)⁺mRNA by hybridization with pBR322-7.2 kilobase pair DNA (plasmid containing the 7.2-kilo base pair genomic fragment), immobilized on a nitrocellulose filter according to the procedures of McKnight *et al.*³². DNA (32.5 μg) was digested with *Eco*RI, the protein extracted with phenol and then precipitated with ethanol before immobilization. Hybridization was carried out in PIPES buffer (950 mM, pH 7.5) containing 50 $\mu\text{g ml}^{-1}$ bean RNA, 2 mM EDTA, 0.4% SDS, 0.5 M NaCl and 30% formamide at 47°C for 2 h with gentle shaking. After being thoroughly washed, the hybridized RNA was eluted from the filter and translated. The products of cell-free translation were analysed as described for A. Lane h, products coded by bean poly(A)⁺ RNA; lane i, products coded by RNA selected by cloned phaseolin DNA; lane j, products made in the absence of added RNA, showing the prominent band (apparent 44,000) observed when ³⁵S-Met is used as substrate in the reticulocyte system.





The solid black areas indicate coding regions that are interrupted by three intervening sequences (white regions), IVS-A, IVS-B and IVS-C. *d*, Map of phaseolin cDNA in clone pBR322.74. The solid black area contains the nucleotide sequence shown in Fig. 5. Complementary DNA was prepared by reverse transcription of 16S bean mRNA²⁴; synthesis of double-stranded cDNA, G-C tailing, and cloning into pBR322 were performed as described by Weinand *et al.*³³. The horizontal arrows shown in *c* and *d* indicate the direction of sequencing for the genomic and cDNA clones, respectively. bp, Base pairs.

Intervening sequences in cloned phaseolin genomic DNA

To compare the sequence of the phaseolin mRNA with that of the cloned phaseolin genomic DNA, a cDNA clone was prepared by reverse transcription of the mRNA template, followed by synthesis of double-stranded DNA with *Escherichia coli* polymerase I, G-C tailing, and insertion into pBR322 by the procedures of Weinand *et al.*³³. cDNA clones obtained were hybridized against the ³²P-labelled 7.2-kilobase genomic DNA fragment; 30 cDNA clones gave positive signals (data not shown) and the strongest hybridizing cDNA clone was selected for further study. A restriction map of this cDNA clone revealed some restriction sites (*Pst*I, *Xba*I, *Sac*I and *Alu*I) in common with the genomic clone (compare Fig. 4*b, d*). Hence, the regions between these sites in both the cDNA and genomic clones were chosen for sequencing by the procedures of Maxam and Gilbert³⁴. The comparison shown in Fig. 5 reveals that they are identical except for three regions present in the genomic DNA but absent from the cDNA. These sequences are presumed to be intervening sequences (introns) that are deleted during processing of the initial nuclear transcript to yield the mature mRNA found in the cytoplasm³⁵. Their relative locations are mapped in Fig. 4*c*.

All three intervening sequences (introns) of this plant gene begin with the nucleotides GT and end with AG, as do those of animal and virus genes³⁶. Recently, Lerner *et al.*³⁷ have compared the regions bounding introns of some 43 eukaryotic DNAs. Their study revealed a general pattern for these regions.

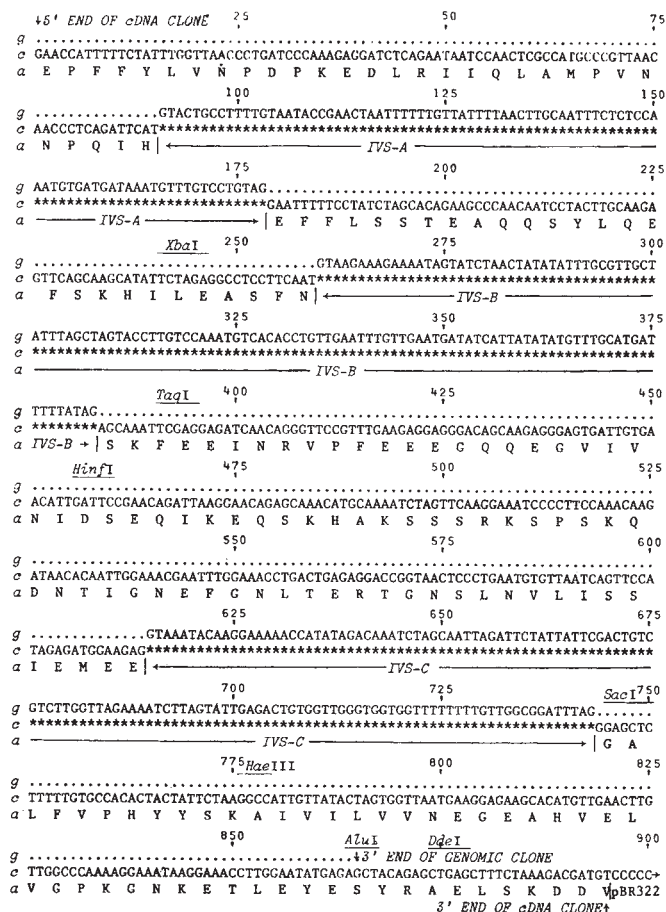


Fig. 5 Comparison of phaseolin genomic and cDNA sequences and the deduced polypeptide sequence. The upper line (g) gives the sequence of the portion of genomic DNA denoted in Fig. 4c. Dots indicate that the genomic sequence is identical to that of the phaseolin cDNA clone shown in the middle line (c). Asterisks denote intervening sequences (*IVS-A*, 88 base pairs; *IVS-B*, 124 base pairs; *IVS-C*, 129 base pairs) that are present in the genomic clone but absent from the cDNA clone. The sequence of 185 amino acids corresponding to the cDNA sequence is shown in the bottom line (a) by the single-letter code⁴⁶. ³²P-end labelling and DNA sequencing were according to the procedure of Maxam and Gilbert³⁴, as modified by Slightom *et al.*²⁰.

	5'exon	Intron	Exon 3'
Consensus sequence	 <u>A</u> <u>G</u> <u>G</u> <u>A</u> <u>A</u> <u>G</u> <u>U</u> <u>U</u> <u>U</u> <u>U</u> <u>U</u> <u>U</u> <u>X</u> <u>C</u> <u>A</u> <u>G</u>		
Phaseolin IVS-A (88 nt)	CAU GUACUG.....UUGUCCUGAG G.....		
Phaseolin IVS-B (124 nt)	AAU GUAAGA.....GAUUUUUUAUAG A.....		
Phaseolin IVS-C (129 nt)	GAG GUAAAU.....GGCGGAUUUAG G.....		

Fig. 6 Comparison of phaseolin intervening sequences with that of a consensus sequence. The consensus sequence shown is from Lerner *et al.*³⁷; single underlines indicate a frequency of occurrence of 45% or greater (in 43 sequences) and double underlines a frequency of 95% or more. Dots under nucleotides (nt) denote differences from the consensus sequence where a purine, X (A or G) replaces a pyrimidine, Y (U or C).

suggesting an involvement in recognition by enzymes responsible for intron excision and exon splicing³⁸. A 'consensus' sequence was derived, and this is compared with the intron-exon boundaries of the phaseolin gene in Fig. 6. For phaseolin introns IVS-A and IVS-B, the boundaries agree closely with the consensus sequence, but 6 of the 11 nucleotides at the 3'-end of IVS-C differ from the generalized sequence. Of these six positions, two purine residues replace pyrimidine locations found in 95% of the sequences analysed by Lerner *et al.*³⁷.

Amino acid sequence from the cloned nucleotide sequences

The DNA sequences were subjected to computer analysis to determine the derived amino acid sequence. Only one open reading frame was found in the DNA sequence of the cDNA clone, and the corresponding amino acid sequence (representing a fragment of about 40% of the length of a phaseolin polypeptide) is shown in Fig. 5. The amino acid composition of this fragment seems to be representative of the overall protein as a good agreement (coefficient of correlation 0.95) was obtained between the derived sequence and that found by amino acid analysis of the native protein³⁹.

Peptide mapping of phaseolin positioned two methionine residues some 12 kilodaltons apart, with one residue located about 6 kilodaltons from one end of the polypeptide⁷. Only two methionine residues were present in the derived sequence shown in Fig. 5 and these are 110 amino acid residues (about 12 kilodaltons) apart. It seems likely, therefore, that the derived sequence starts some 6 kilodaltons from the N-terminus of the phaseolin polypeptide. The peptide mapping data⁷ also showed that the phaseolin polypeptides have an essentially identical amino acid sequence for some 60% of their length; the heterologous region lying to the C-terminal (direction deduced from the present data) end of the molecules is presumed to be

primarily responsible for the multiple phaseolin polypeptides resolved in high-resolution two-dimensional electrophoresis⁹. The cloned DNA fragments are located within the homologous region of phaseolin. Therefore, the correspondence of the genomic and cDNA sequences, and also the cross-hybridization to multiple phaseolin mRNAs (as evidenced by the hybrid-selection and hybrid-arrest data), are not surprising.

Interestingly the derived sequence reveals that 13 of the 19 aspartate residues are asparagine and of the 36 glutamate residues, 10 are glutamine; that is, there are 23 amide residues in the defined sequence of 185 amino acids. The high amide content of phaseolin reflects its role as a N-storage protein for the seed, but a high proportion of acidic residues is also expected from the general properties of this protein.

The sequence CG is rarely found in either the first or second (absent, but expected four times by random chance) or second and third (occurs once; random expectation is seven times) codon positions. Thus, of the six arginine codons available, only two are used to an appreciable extent in the sequence analysed thus far.

Conclusions

From the data available, most of the region coding for phaseolin probably lies within the area denoted by the heavy black line in Fig. 4a, b. The remainder is presumed to be within the stippled regions at each side of this area because they show weaker hybridization to the phaseolin probe and because no hybridization occurs outside these regions. Thus, it seems that clone Ch24A-177.4 contains an entire phaseolin gene, together with extensive sequences flanking its 3' and 5' ends.

Our results show that the structure of this plant gene is similar to that of animal DNAs in containing intervening sequences. This isolation of one of the genes encoding the major storage globulin in bean seeds should provide a basis for exploring regulatory constraints for the expression of this nutritionally significant protein.

We thank Dr O. Smithies (NIH grant GM-20069) and his associates, especially Tom Szeto, Ann Blechl and Natalie Borenstein, for guidance in cloning operations and for the use of his P2 facility in which all recombinant operations were carried out according to current NIH guidelines. We also thank Dr W. M. Fitch for the computer derivation of the amino acid code from the nucleotide sequence and Dr J. Ross for advice and a gift of S₁ nuclease. Reverse transcriptase was obtained from Dr J. W. Beard of Life Sciences through the Viral Cancer Program of the NCI and the Charon 24A was a gift from F. R. Blattner. This study was supported by grants from USDA/SEA (5901-0410-9-0357-0), NSF (PCM 78-11804), the Herman Frash Foundation and by the Research Division of the College of Agricultural and Life Sciences (Hatch 1482) of the UW-Madison.

Received 25 July; accepted 24 October 1980.

- Osborne, T. B. *J. Am. chem. Soc.* **16**, 633-764 (1894).
- McLeester, R. C., Hall, T. C., Sun, S. M. & Bliss, F. A. *Phytochemistry* **12**, 85-93 (1973).
- Sun, S. M., McLeester, R. C., Bliss, F. A. & Hall, T. C. *J. biol. Chem.* **249**, 2118-2121 (1974).
- Pusztai, A. & Watt, W. B. *Biochim. biophys. Acta* **207**, 413-431 (1970).
- Joubert, F. J. *S. Afr. Chem. Inst.* **10**, 16-20 (1957).
- Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. L. *J. biol. Chem.* **252**, 1102-1106 (1977).
- Ma, Y., Bliss, F. A. & Hall, T. C. *Pl. Physiol.* **66**, 897-902 (1980).
- O'Farrell, P. H. *J. biol. Chem.* **250**, 4007-4021 (1975).
- Brown, J. W. S., Bliss, F. A. & Hall, T. C. *Pl. Physiol.* **6**, 838-840 (1980).
- Viotti, A. *et al. Eur. J. Biochem.* **102**, 211-222 (1979).
- Park, W. D., Lewis, E. D. & Rubenstein, I. *Pl. Physiol.* **65**, 98-106 (1980).
- Larkins, B. A. *et al. in Genome Organization and Expression in Plants* (ed. Leaver, C. J.) 203-217 (Plenum, New York, 1980).
- Beachy, R. N., Barton, K. A., Madison, J. T., Thompson, J. F. & Jarvis, N. in *Genome Organization and Expression in Plants* (ed. Leaver, C. J.) 273-281 (Plenum, New York, 1980).
- Moreira, M. A., Hermanson, M. A., Larkins, B. A. & Nielsen, N. C. *J. biol. Chem.* **254**, 9921-9926 (1979).
- Sun, S. M., Mutschler, M. A., Bliss, F. A. & Hall, T. C. *Pl. Physiol.* **61**, 918-923 (1978).
- Mutschler, M. A., Bliss, F. A. & Hall, T. C. *Pl. Physiol.* **65**, 627-630 (1980).
- Hall, T. C. *et al. in The Plant Seed* (eds Rubenstein, I., Phillips, R. L., Green, C. E. & Gengenbach, B. G.) 3-25 (Academic, New York, 1979).
- Bliss, F. A. & Hall, T. C. *Cereal Foods World* **22**, 106-113 (1977).
- Blattner, F. R. *et al. Science* **202**, 1279-1284 (1978).
- Slightom, J. L., Blechl, A. E. & Smithies, O. *Cell* **21**, 627-638 (1980).
- Maniatis, T. *et al. Cell* **15**, 687-701 (1978).
- Blin, N. & Stafford, D. W. *Nucleic Acids Res.* **3**, 2303-2308 (1976).

- Hall, T. C. *et al. in Genome Organization and Expression in Plants* (ed. Leaver, C. J.) 259-272 (Plenum, New York, 1980).
- Kacian, D. L. & Myers, J. C. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2191-2195 (1976).
- Moore, D. D., Denniston-Thompson, K., Furth, M. E., Williams, B. G. & Blattner, F. R. *Science* **198**, 1041-1046 (1977).
- Bolivar, F. *et al. Gene* **2**, 95-113 (1977).
- Hall, T. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3196-3200 (1978).
- Alwine, J. C., Kemp, D. J. & Stark, G. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5350-5354 (1977).
- Rave, N., Crkvenjakov, R. & Boedtker, H. *Nucleic Acids Res.* **6**, 3559-3567 (1979).
- Maniatis, T., Jeffrey, A. & Kleid, D. G. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1184-1188 (1975).
- Paterson, B. M., Roberts, B. Z. & Kuff, E. L. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4370-4374 (1977).
- McKnight, G. S., Lee, D. C., Mayo, K. E. & Palmiter, R. *Nucleic Acids Res.* **6**, 2435-2452 (1979).
- Wienand, U., Bruschke, C. & Feix, G. *Nucleic Acids Res.* **6**, 2707-2715 (1979).
- Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560-565 (1977).
- Tilghman, S. N. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 725-729 (1978).
- Breathnach, R., Benoist, C., O'Hare, K., Gannon, F. & Chambon, P. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4853-4857 (1978).
- Lerner, M. R., Boyle, J. A., Mount, S. M., Wolin, S. L. & Steitz, J. A. *Nature* **283**, 220-224 (1980).
- Rogers, J. & Wall, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1877-1879 (1980).
- Sun, S. M. thesis, Univ. Wisconsin, Madison (1974).
- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Buchbinder, B. U. thesis, Univ. Wisconsin, Madison (1980).
- Brakke, M. K. & Van Pelt, N. *Analyst. Biochem.* **38**, 56-64 (1970).
- Laemmli, U. K. *Nature* **227**, 680-685 (1970).
- Bonner, W. M. & Laskey, R. A. *Eur. J. Biochem.* **46**, 83-88 (1974).
- Fyrberg, E. A., Kindle, K. L., Davidson, N. & Sodja, A. *Cell* **19**, 365-378 (1980).
- IUPAC-IUB Commission *Biochem. J.* **113**, 1-4 (1969).

LETTERS

Spectral evolution of the 5 March 1979 γ burst

E. E. Fenimore, W. D. Evans, R. W. Klebesadel, J. G. Laros & J. Terrell

University of California, Los Alamos Scientific Laboratory, Los Alamos, New Mexico 87545

The γ burst¹ of 5 March 1979 was observed by nine experiments²⁻⁶ widely spaced on an interplanetary scale allowing an accurate position for a γ -burst source to be determined^{2,6} for the first time. Spectral observations in the range 50–1,000 keV had sufficient time resolution to resolve spectral changes within the main burst. Early in the main burst, the typical photon energies declined from a spectrum similar to a 30 keV black body to that of a ~ 26 keV black body. Thereafter, at the shoulder on the main burst the spectrum became harder ($KT \sim 31$ keV). The γ burst apparently triggered an X-ray pulsar with a period of ~ 8.0 s (refs 3–5, 7) and with a softer spectrum ($KT \sim 12$ keV). We show here that, although the source is probably a neutron star with an obliquely rotating magnetic field, simple cooling of residual hot spots does not completely explain the pulsations.

Figure 1 presents the time history and spectral development of the 5 March 1979 γ -burst as observed by the Pioneer Venus Orbiter (PVO) detector (see ref. 8 for instrumental details). The PVO instrument varies its sampling rate depending on the count rate, which was near saturation during the burst. As a result, the intensity and spectra were recorded at the highest sampling rate of any of the satellites observing the event (either every 0.25 ms or 0.5 ms for the intensity and every ~ 6 ms for the spectra). Figure 1a, b presents the intensity and a hardness ratio for the burst phase of the event, Fig. 1c, d shows the intensity and hardness ratio plotted on a scale intended to display the 8-s periodicity^{3-5,7}. The intensities and the hardness ratios in Fig. 1 have been binned to improve the statistics.

The hardness ratio is defined to be the ratio of the sum of (background-corrected) counts in three channels spanning 100–1,000 keV to the counts in the 50–100 keV channel. Each ratio is represented by a box whose width is the range of time of the sample and whose height is determined by $\pm 1.0\sigma$ error limits. By folding a range of black-body spectra with the instrumental response, this ratio has been mapped into an effective black-body temperature at the right-hand side of Fig. 1d. A black-body form was used to parameterize the data in a manner which allows an estimate of the size of the emitting region by Stefan's law.

A key feature of this burst is its very fast rise time. A reanalysis⁷ of the PVO data reveals that rise times of ≤ 1 ms are consistent with the data. (This value is larger than our original² value of 0.25 ms.) After the sharp rise, the intensity continues to rise for ~ 25 ms. During this rise and until $t = 75$ ms the hardness ratio has a downward trend. Black bodies with KT from 30 to 26 keV can reproduce the observed hardness ratio during this phase. However, the observed distribution of counts has too many counts in the two higher energy bins (250–500 and 500–1,000 keV) to be entirely consistent with black-body radiation. Thus, during this phase, the hardness ratio and the effective KT should be used only as an indicator of typical energies involved. Starting at ~ 80 ms after the initial rise, there is a shoulder on the light curve of the main burst which lasted for ~ 0.1 s. This shoulder has a tendency to have a somewhat harder spectrum (~ 31 keV).

The significance of these variations was checked by χ^2 tests. Two ranges were tested, from $t = 0.0$ to 75 ms and from $t = 0.0$

to 120 ms. Random fluctuations could account for the observed distribution of hardness ratios only 3% and 2% of the time, respectively. We conclude that there is a statistically significant trend for the spectrum to soften initially (until $t \sim 75$ ms) and then harden during the shoulder on the γ -burst light curve. In contrast to the first 80 ms, the spectrum during the shoulder can be fit by a black-body spectrum (among other spectral forms) to within the accuracy of the statistics. As the intensity within the shoulder decays to near background (~ 150 c.p.s. (counts per s) at $t \sim 2.0$ s), the spectrum softens dramatically.

Following the γ -burst, the source exhibited many of the characteristics of a hard X-ray pulsar³. The period was observed to be 8.0 ± 0.1 (refs 3–5, 7). Figure 1c shows the time history for the first few cycles of the pulsations. (For longer time histories where the periodicity is more evident see refs 3–5, 7). Note that, particularly for the first pulse, the hardness ratio varies in phase with the intensity from near zero between the pulses to a KT_{eff} of ~ 12 keV at the peak of the pulse (see Fig. 1d). This statistically significant variation in phase with the intensity is probably due to a varying look angle to the source as the object rotates. Due to deteriorating statistics and a greater contribution of the interpulse, it is not possible to determine whether similar spectral changes occur in the subsequent pulses.

The short rise time, the pulse period, the regularity of the pulses, the existence of an interpulse, and the observation of a line at a location consistent with the 511-keV line³ redshifted by a 1 solar-mass neutron star, all argue that the pulsations are due to hot spots at the magnetic poles of a rotating neutron star. If so, a possible explanation for the difference between the γ -burst temperature and the X-ray pulsar temperature is that the X-ray pulsar is due to residual hot spots at the poles which are cooling.

If the observed lower temperature ($KT \sim 12$ keV) in the pulses represents a cooling from the initial hotter γ -burst, one would expect the side of the star giving the γ -burst to show a continually decreasing temperature and count rate. Figure 2

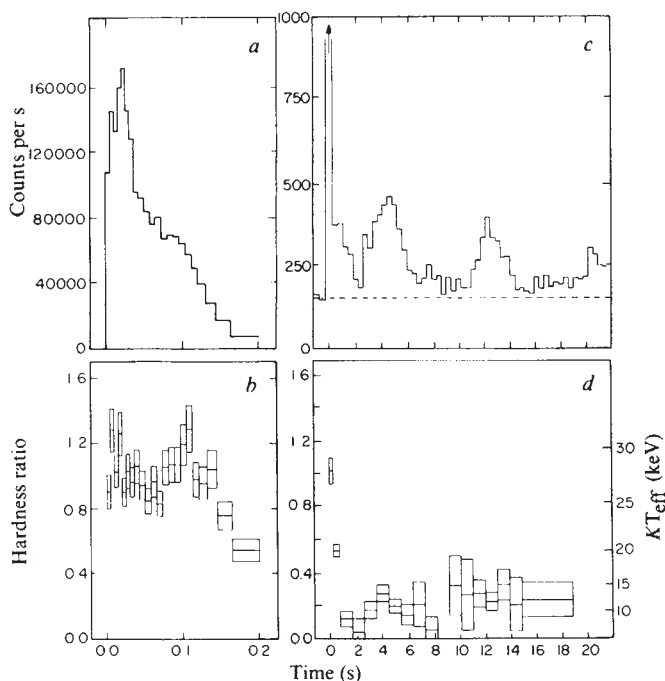


Fig. 1 The Pioneer Venus Orbiter time history and hardness ratio for the 5 March 1979 γ -burst. Initial time t_0 is at 57118.019 s UT. The dotted line in c is the background level. a, b, Burst; c, d, 8-s pulsation.

contrasts the behaviour of the two sides of the star and is based on the Venera 11 and 12 observations³ because they had the highest sensitivity and were made from three-axis stabilized satellites. Each point on the histogram represents the average intensity (scaled to the PVO sensitivity) near the peak of a pulse, determined by summing the Venera 11 and 12 observations³ for 2 s. The cross-hatched regions represent the pole on the side of the star from which the γ -burst was observed. The diagonally striped regions are due to the opposite pole.

The flux from the side away from the burst is consistent with cooling (that is, it is decreasing). However, the pole associated with the burst shows an increasing average count rate, being 95 ± 1.57 c.p.s. for the pulse at ~ 8 s and 113 ± 1.7 for the pulse at $t \sim 16$ s. Applying a standard statistical test, one finds that the difference in count rate represents an $\sim 10\sigma$ deviation. We conclude that, unless the Venera data contains nonstatistical uncertainties, the 2-s averaged count rate from the pole associated with the burst increased between $t \sim 8$ and ~ 16 s.

Figure 1d shows how the spectrum changed between these two intervals. From $t \sim 7.0$ to 8.25 s, the hardness ratio was ~ 0.05 . During the period $t \sim 8.25$ – 9.25 s, the high energy channels (100–1,000 keV) were below anticipated background, implying a hardness ratio of zero. In contrast to these values, the interval from $t \sim 15.0$ to 18.75 s had a hardness ratio of 0.23. The increase in the observed count rate in the higher energy channels between the two pulses is statistically significant at the 98% confidence level. Also, according to the PVO data the overall observed count rate significantly decreased (5.8σ), implying that the temperature was indeed increasing while either the source size was decreasing or the column density was increasing. However, the decrease in the PVO overall count rate is not in accord with the increase observed from the three-axis stabilized observations of the Veneras. The PVO observations were possibly affected by spin modulation of the satellite. Although the spin axis was pointed to within 3 degrees of the location of the burst^{2,6}, there could have been sufficient material moving in and out of the field of view to cause the small discrepancy between PVO and the Veneras. In either case (if the intensity increased as observed by the Veneras or if the hardness ratio increased as observed by PVO), the cooling explanation is unlikely. Because the PVO had a possible (although unlikely) source of systematic error, we are inclined to accept the Venera result that the intensity increased. If so, the increase in hardness ratio observed by PVO is less certain.

One possibility consistent with the observations is that the γ -burst altered the magnetosphere, triggering a short-lived (few minutes), accretion-driven, hard X-ray pulsar. The difference in hardness ratio between the γ -burst and the pulsation then is a result of their being produced by separate but related energy sources. One constraint on such a model would be whether the magnetosphere can hold sufficient material without violating the observed upper limit on the steady state X-ray flux⁹. In addition the source would have to be close enough to avoid violating the Eddington limit¹⁰, ruling out N49 as the counterpart. A similar explanation is that the neutron star suffered a collision with a nearly intact comet (as recently proposed in ref. 11 for the 5 March γ -burst) with the pulsations then being due to left over debris accreting down the field lines.

An accreting model for the pulses is not the only possible explanation. For example, if the initial γ rays were collimated into a beam that was not colinear with our line of sight to the pulsar, and if the beam subsequently broadened between $t \sim 8$ s and ~ 16 s, then the average count rate could increase even if the source were cooling. Indeed, ref. 3 shows that the pulse at $t \sim 8$ s was probably narrower than subsequent pulses, contributing to its lower average count rate. There must be other possible beam geometries that could have a similar effect.

The nature of the γ -burst remains uncertain. We will not consider magnetospheric accretion because current models^{12,13} predict rise times that are 100–1,000 times larger than observed for the γ -burst. Because both thermonuclear flashes^{14,15} and internal restructuring^{16,17} probably originate beneath the outer

surface of the neutron star but propagate to the surface in a similar manner, they might be expected to have similar characteristics. However, other features of the source might be used to distinguish between them. In particular, a successful model must be able to function in the presence of a magnetic field, be able to turn on a hard X-ray pulsar, and give recurring bursts with a dynamic range of 100 (refs 3, 18).

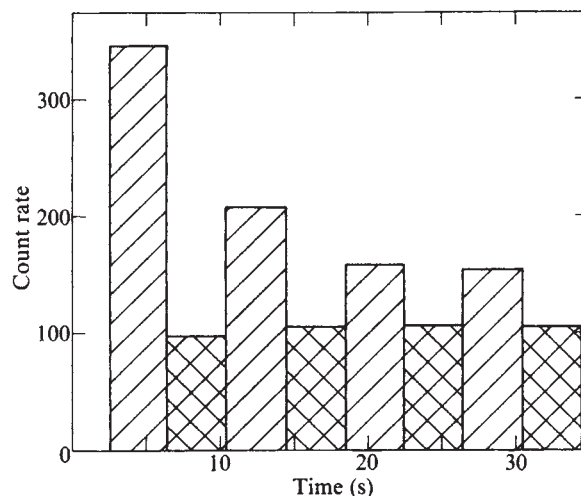


Fig. 2 Average count rate for the X-ray pulses obtained by summing the Venera 11 and 12 observation over 2-s periods (see ref. 3), normalized to the PVO count rate. The cross-hatched regions represent the side of the star associated with the γ -burst and the diagonally-striped regions represent the opposite side.

In the context of internal restructuring, a spin axis different from the magnetic axis (as implied by the pulsations) can apply external torques to the crust of the neutron star which builds up strain even for a slowly rotating neutron star (see review in ref. 19). The resulting starquake would alter the magnetosphere, perhaps providing the short-lived instability driving the X-ray pulsar by accretion. Current models of thermonuclear flashes have reached the quantitative stage for unmagnetized stars but not for magnetized stars. It has been suggested^{15,20} that a magnetic field inhibits the flashes and that is why X-ray bursters show no sign of rotational modulation¹⁵. Because neither internal restructuring models nor magnetized neutron star models have yet reached a stage where quantitative comparisons can be made, the mechanism of the 5 March 1979 γ -burst remains uncertain.

We thank A. Cox, M. Newman, A. Petschek and J. Solem for helpful discussions. This work was funded jointly by NASA and the US Department of Energy.

Received 2 September; accepted 20 October 1980.

- Klebesadel, R. W., Strong, I. B. & Olson, R. A. *Astrophys. J. Lett.* **182**, L85–88 (1973).
- Evans, W. D. *et al.* *IAU Circ. No.* 3356 (1979).
- Mazets, E. P. *et al.* *Nature* **282**, 587–589 (1979).
- Barat, C. *et al.* *Astr. Astrophys.* **79**, L24–25 (1979).
- Cline, T. L. *et al.* *Astrophys. J. Lett.* **237**, L1–6 (1980).
- Evans, W. D. *et al.* *Astrophys. J. Lett.* **237**, L7–10 (1980).
- Terrell, J., Evans, W. D., Klebesadel, R. W. & Laros, J. G. *et al.* *Nature* **285**, 383–385 (1980).
- Evans, W. D. *et al.* *Science* **205**, 119–121 (1979).
- Helford, D. J. & Long, S. L. *Nature* **282**, 589–591 (1979).
- Petschek, A. & Colvin, J. D. *Astrophys. J. Lett.* (submitted).
- Newman, M. J. & Cox, A. N. *Astrophys. J.* (submitted).
- Lamb, D. Q. *et al.* *Nature phys. Sci.* **246**, 52–54 (1973).
- Lamb, D. Q. & Lamb, F. K. *Ann. NY Acad. Sci.* **302**, 261–299 (1977).
- Woosley, S. E. & Taam, R. E. *Nature* **263**, 101–103 (1976).
- Joss, P. C. *Nature* **270**, 310–314 (1977); *Astrophys. J. Lett.* **225**, L123–127 (1978).
- Pacini, F. & Ruderman, M. *Nature* **251**, 399–400 (1974).
- Fabian, A. C. *et al.* *Astrophys. Space Sci.* **42**, 77–81 (1976).
- Mazets, E. P. & Golenetskii, S. V. *Recent Results of the Gamma-Ray Burst Studies in the Konus Experiment* (A. F. Ioffe Physico-Technical Institute Preprint 632, 1979).
- Ruderman, M. A. *Rev. Astr. Astrophys.* **10**, 427–476 (1972).
- Joss, P. C. & Li, F. K., *Astrophys. J.* **238**, 287–295 (1980).

Is the light cylinder the site of emission in pulsars?

Houshang Ardavan

Institute of Astronomy, University of Cambridge, Madingley Road, Cambridge CB3 0HA, UK

There is no agreement in reports on the oblique-rotator model of pulsars¹⁻⁹ as to the site of emission within the pulsar magnetosphere. It is generally assumed, however, that the plasma-electromagnetic fields constituting the magnetosphere obey the quasistatic constraint $\partial f / \partial t = -\omega_0 \partial f / \partial \varphi$, where ω_0 is the angular frequency of rotation of the central neutron star, t the time, and the cylindrical coordinates (r, φ, z) refer to an inertial frame whose z -axis is coincident with the axis of rotation of the star. Accordingly, the pulsar emission is attributed to a source whose distribution has a rigidly rotating pattern; though, of course, none of the charged particles which constitute the source and provide the medium for the propagation of the pattern associated with the source, are themselves constrained to be co-rotating. Here, the spectral distribution of the power emitted by such a rotating pattern is calculated and it is shown that electromagnetic radiation of frequency $\omega \gg \omega_0$, the observed radiation from pulsars, is produced predominantly by the electric currents at the light cylinder $r = c/\omega_0$ of the magnetosphere. This is a model-independent result; it is valid irrespective of the type of emission mechanism and applies to all previously proposed models of the magnetosphere which are quasistatic and in which the radial length scale of the plasma distribution is of the order of c/ω_0 .

From the retarded solution of the inhomogeneous Maxwell's equations it follows¹⁰ that the energy radiated per unit frequency and per unit solid angle by any localized distribution of electric current is

$$\frac{dI(\omega)}{d\Omega} = \frac{\omega^2}{4\pi^2 c^3} \left| \int dt \int d^3x \hat{n} \times [\hat{n} \times \mathbf{j}(\mathbf{x}, t)] \exp[i\omega(t - \hat{n} \cdot \mathbf{x}/c)] \right|^2 \quad (1)$$

where $\mathbf{j}$ is the current density, $\hat{n}$ is the unit vector directed from the position of the source to the observation point, and the domain of integration extends over all values of the space-time coordinates $\mathbf{x}$ and t . In the inertial coordinate system whose origin is coincident with the position of the neutron star and whose z -axis lies along the rotation axis $\hat{k}$, an arbitrarily fixed direction of $\hat{n}$ may be designated by the spherical angles (θ_0, φ_0) where $\theta_0 = \cos^{-1}(\hat{n} \cdot \hat{k})$. If in addition the vector $\mathbf{j}$ is decomposed into its cylindrical components (j_r, j_φ, j_z) and $\mathbf{x}$ is expressed in terms of (r, φ, z) , then equation (1) assumes the form

$$\frac{dI(\omega)}{d\Omega_0} = \frac{\omega^2}{4\pi^2 c^3} |A_{\parallel}(\omega, \theta_0, \varphi_0) \hat{e}_{\parallel} + A_{\perp}(\omega, \theta_0, \varphi_0) \hat{e}_{\perp}|^2 \quad (2)$$

in which

$$A_{\parallel, \perp} = \int dt \int r dr dz d\varphi j_{\parallel, \perp}(r, \varphi, z, t) \times \exp \left\{ i\omega \left[t - \frac{r}{c} \sin \theta_0 \cos(\varphi - \varphi_0) - \frac{z}{c} \cos \theta_0 \right] \right\} \quad (3)$$

$$j_{\parallel} = j_r \sin(\varphi - \varphi_0) + j_\varphi \cos(\varphi - \varphi_0) \quad (4)$$

$$j_{\perp} = \cos \theta_0 [j_\varphi \sin(\varphi - \varphi_0) - j_r \cos(\varphi - \varphi_0)] + j_z \sin \theta_0 \quad (5)$$

and the directions $\parallel$ and $\perp$ are defined by $\hat{e}_{\parallel} = (\hat{k} \times \hat{n}) / |\hat{k} \times \hat{n}|$ and $\hat{e}_{\perp} = \hat{n} \times \hat{e}_{\parallel}$.

The quasistatic constraint can now be introduced by requiring the cylindrical components of the current density to be functions of φ and t in the combination $\varphi - \omega_0 t \equiv \hat{\varphi}$ only (see refs 1, 11), that is by writing

$$j_{r, \varphi, z}(r, \varphi, z, t) = j_{r, \varphi, z}(r, z, \hat{\varphi}) = \int_{-\infty}^{+\infty} j_{r, \varphi, z}(r, z, \hat{\varphi}') \delta(\hat{\varphi}' - \hat{\varphi}) d\hat{\varphi}' \quad (6)$$

The second part of equation (6) is no more than a mathematical identity in view of the definition of the Dirac delta function. It provides a useful representation of the quasistatic functions (j_r, j_φ, j_z) , however, because by making the integrals over φ and t in equation (3) independent of $\hat{\varphi}$, this representation enables us to derive general expressions for $A_{\parallel}$ and $A_{\perp}$ in which the two variables φ and t are everywhere replaced by the single variable $\hat{\varphi}' = \varphi' - \omega_0 t'$. In the case of $A_{\parallel}$, for instance, once equation (6) is inserted in equation (3) the integration over t may be immediately performed to obtain

$$A_{\parallel} = \frac{1}{\omega_0} \exp \left(i \frac{\omega}{\omega_0} \varphi_0 \right) \int r dr dz d\hat{\varphi}' \times \exp \left[-i \frac{\omega}{\omega_0} \left(\hat{\varphi}' + \frac{z\omega_0}{c} \cos \theta_0 \right) \right] \left\{ j_r(r, z, \hat{\varphi}') \int_0^{2\pi} d\sigma \sin \sigma \times \exp \left[i \frac{\omega}{\omega_0} \left(\sigma - \frac{r\omega_0}{c} \sin \theta_0 \cos \sigma \right) \right] + j_\varphi(r, z, \hat{\varphi}') \int_0^{2\pi} d\sigma \cos \sigma \times \exp \left[i \frac{\omega}{\omega_0} \left(\sigma - \frac{r\omega_0}{c} \sin \theta_0 \cos \sigma \right) \right] \right\} \quad (7)$$

where $\sigma \equiv \varphi - \varphi_0$. The integrals over σ can also be carried out explicitly. They are non-zero only when the ratio ω/ω_0 is an integer (ref. 10, p. 96), and are expressible in terms of Bessel functions of integer order m when $\omega = m\omega_0$ (ref. 12, p. 209). Because $j(r, z, \hat{\varphi})$ is left unchanged whether φ is increased by 2π or equivalently t is decreased by $2\pi/\omega_0$, it is not unexpected that the spectral distribution of the radiation should in the present case be discrete.

The quasistatic versions of $A_{\parallel}$ and $A_{\perp}$ thus obtained, when inserted in equation (2), yield the following expression for the angular distribution of power radiated into the m th harmonic:

$$\frac{dP_m}{d\Omega} = \frac{m^2 \omega_0^2}{2\pi c^3} |C_{\parallel}(m, \theta) \hat{e}_{\parallel} - C_{\perp}(m, \theta) \hat{e}_{\perp}|^2 \quad (8)$$

where

$$C_{\parallel} = \int r dr dz d\hat{\varphi} \exp \left[-im \left(\hat{\varphi} + \frac{z\omega_0}{c} \cos \theta \right) \right] \times \left[j_\varphi \frac{dJ_m(m\xi)}{d(m\xi)} + \frac{i}{\xi} j_r J_m(m\xi) \right] \quad (9)$$

$$C_{\perp} = \int r dr dz d\hat{\varphi} \exp \left[-im \left(\hat{\varphi} + \frac{z\omega_0}{c} \cos \theta \right) \right] \times \left[j_r \cos \theta \frac{dJ_m(m\xi)}{d(m\xi)} - i \left(\frac{\cos \theta}{\xi} j_\varphi - \sin \theta j_z \right) J_m(m\xi) \right] \quad (10)$$

and

$$\xi \equiv \frac{r\omega_0}{c} \sin \theta$$

Here, $\hat{\varphi}'$ and θ_0 have been written $\hat{\varphi}$ and θ for brevity, and $dI/d\Omega$ has been replaced by $(2\pi/\omega_0^2)(dP_m/d\Omega)$ to convert energy per unit frequency to power per harmonic. Note that j_r, j_φ and j_z are functions of $(r, z, \hat{\varphi})$, and that the integrals extend over all values of r, z and $\hat{\varphi}$ for which the current density is non-zero. It is easy to see that when specialized to the case of a point source for which $j_r = j_z = 0$, equation (8) is the same as the corresponding expression for synchrotron radiation¹².

In the range of frequencies $m \gg 1$, asymptotic values of the Bessel function appearing in equation (8) are given by¹³

$$J_m(m \operatorname{sech} \alpha) \sim (2\pi m \tanh \alpha)^{-1/2} \exp[m(\tanh \alpha - \alpha)] \quad (11)$$

or

$$J_m(m \sec \beta) \sim (\frac{1}{2}\pi m \tan \beta)^{-1/2} \cos[m(\tan \beta - \beta) - \frac{1}{4}\pi] \quad (12)$$

depending on whether ξ is greater or less than one. When the current density has limited total fluctuation in the radial direction, therefore, the rapid oscillation of the Bessel function in the interval $\xi > 1$ renders the magnitude of the power originating from $(r\omega_0/c) \sin \theta > 1$ negligible. According to the principle of stationary phase, the main contributions to the values of the integrals in equation (8) for $\xi > 1$ come from the neighbourhood of the surface where

$$\frac{d}{d\beta} [m(\tan \beta - \beta) - \frac{1}{4}\pi] = m \left[\left(\frac{r\omega_0}{c} \sin \theta \right)^2 - 1 \right] = 0 \quad (13)$$

A similar conclusion in fact follows from the behaviour of the Bessel function in $\xi < 1$. Equation (11) in conjunction with the asymptotic value of the Bessel function at $\xi = 1$ (ref. 13) shows that the ratio

$$\frac{J_m(m \operatorname{sech} \alpha)}{J_m(m)} \sim \frac{3^{2/3} \Gamma(\frac{2}{3})}{2^{1/3} (2\pi)^{1/2}} \frac{\exp [m(\tanh \alpha - \alpha)]}{m^{1/6} \tanh \alpha} \quad (14)$$

has a sharp peak in the neighbourhood of $(r\omega_0/c) \sin \theta = 1$. In this case, the order of magnitude $m^{-1/6} \exp(-\alpha m)$ of this ratio for $\alpha \gg 1$, that is for $r\omega_0/c \ll 1$, is small for the same mathematical reason that the power radiated by a particle in the non-relativistic as compared with that in the extreme relativistic regime is small. This mathematical similarity is, of course, not fortuitous (see ref. 11).

Equation (8) also shows that for a current density whose length scale of variation along z is c/ω_0 , the main contribution to the values of $C_{\parallel}$ and $C_{\perp}$ is received from directions where $m \cos \theta \approx 1$, because the integrals over z simply represent the Fourier amplitudes of the current density corresponding to the wave number $m\omega_0 \cos \theta/c$. This beaming of the high-frequency radiation into $\theta \approx \pi/2$, in turn implies that ξ attains the value unity in the neighbourhood of the light cylinder. Therefore I suggest that the higher the frequency, the closer the site of emission to the light cylinder. Because the quasistatic condition is associated with a rotating pattern for the source (see ref. 11 for a detailed discussion), it is not too surprising that the emission from inside the light cylinder should be much smaller than that from the vicinity of the light cylinder; the more unexpected outcome of the present analysis is that the emission from beyond the light cylinder is also relatively small.

Because it follows from a formalism in which the electric current is left unspecified, the present result is clearly independent of both the details of the mechanism by which the radiation is emitted and of the effects of the plasma through which it is propagated. The structure of the magnetosphere, moreover, has a bearing on this result only in that the plasma distribution is required to have a length scale of the order of c/ω_0 . The validity of the quasistatic condition is, of course, implicitly dependent on the existence of an agent—such as a large scale magnetic field—which affects the coupling between the neutron star and the magnetosphere. However, imposing the quasistatic constraint can also be justified on independent observational grounds¹¹.

The present analysis clearly establishes that there is an inconsistency in those models which adopt the quasistatic constraint and at the same time identify the surface of the neutron star as the site of emission. According to equation (14), the power radiated into the m th harmonic $\omega = m\omega_0$ receives a contribution from the electric currents in the vicinity of the light cylinder which is by a factor of the order of $m^{1/3} \exp(2\alpha m)$ greater than that received from the vicinity of the neutron star. Thus, even for radio waves with $m \approx 10^8$, the observed emission originates almost exclusively from the light cylinder when the electric currents are distributed throughout the magnetosphere.

The present analysis has centred on only one particular implication of equation (8), but in fact this equation, which incorporates the coherent character of the radiation automatically, also constitutes the departure point for a study of the polarization and the spectrum of the pulsar emission.

Received 11 August; accepted 6 November 1980.

1. Mestel, L. *Nature phys. Sci.* **233**, 149–152 (1971).
2. Cohen, J. M. & Rosenblum, A. *Astrophys. Space Sci.* **16**, 130–136 (1972).
3. Roberts, D. H. & Sturrock, P. A. *Astrophys. J.* **181**, 161–180 (1973).
4. Edean, V. G. *Astrophys. J.* **187**, 359–360 (1974).
5. Henriksen, R. N. & Norton, J. A. *Astrophys. J.* **201**, 719–728 (1975).
6. Mestel, L., Wright, G. A. E. & Westfold, K. C. *Mon. Not. R. astr. Soc.* **175**, 257–278 (1976).
7. Cheng, A. F. & Ruderman, M. A. *Astrophys. J.* **212**, 800–806 (1977).
8. Buckley, R. *Mon. Not. R. astr. Soc.* **183**, 771–778 (1978).
9. Arons, J. & Scharlemann, E. T. *Astrophys. J.* **231**, 854–879 (1979).
10. Jackson, J. D. *Classical Electrodynamics* (Wiley, New York, 1962).
11. Ardavan, H. *Astrophys. J.* (submitted).
12. Landau, L. D. & Lifshitz, E. M. *The Classical Theory of Fields* (Pergamon, Oxford, 1962).
13. Abramowitz, M. & Stegun, I. A. *Handbook of Mathematical Functions* (Dover, New York, 1965).

Photovoltaic properties of merocyanine solid-state photocells

G. A. Chamberlain & P. J. Cooney

Shell Research Ltd, Thornton Research Centre, PO Box 1, Chester CH1 3SH, UK

S. Dennison

Department of Physical Chemistry, Lensfield Road, University of Cambridge, Cambridge CB2 1EP, UK

Considerable progress has been made in improving the sunlight conversion efficiency of solid-state organic photovoltaic cells. Ghosh *et al.*¹ report a 0.7% efficiency for a merocyanine dye Schottky barrier cell. We expect that improvements in energy conversion efficiency may follow if merocyanines of low ionization potential are used in photovoltaic cells, in which the dye layer contains a strongly electronegative dopant such as iodine. To test these ideas we report here the photovoltaic quantum yields and sunlight conversion efficiencies of a series of merocyanines in which the molecular properties have been varied systematically.

Ghosh *et al.*¹ used an evaporated thin film of the dye, sandwiched between thin Al/Al₂O₃ and silver layers and illuminated through the Al contact for maximum response. They proposed that charge carriers are generated by electron transfer from an exciton to unoccupied metal orbitals or oxide surface states at the aluminium/dye interface. The magnitude of the photocurrent at a given light intensity is then governed by the field dependence of geminate recombination between the transferred electron on the aluminium and its positive image charge in the dye layer. We have observed, however, that the photovoltaic response of this type of cell is extremely sensitive to the presence of added gases, for example, ambient air and particularly iodine vapour. By preparing and testing the cell in a vacuum of 10⁻⁶ torr we have found that the magnitude of the photovoltaic response increases by several powers of 10 when the dye is doped with iodine vapour; the dark conductivity of the dye, however, is unchanged. It seems likely, therefore, that charge carriers may be photogenerated by a charge transfer process from excited dye to iodine, possibly through an exciplex precursor. The undesirable back electron transfer reaction must then compete with charge carrier separation by hopping, which occurs in the built-in field in the region of the aluminium/dye interface. The extent of charge transfer from a donor (the dye) to an acceptor (iodine) in the ground and excited states of the complex increases as the ionization potential of the donor decreases².

In general terms³, a merocyanine dye consists of an electron-donating group (the donor) linked by a conjugated polymethine bridge to an electron-accepting group (the acceptor). The intramolecular charge transfer that occurs in the ground and excited states depends on the relative strengths of the donor and acceptor groups. In addition, the stability and lifetime of the

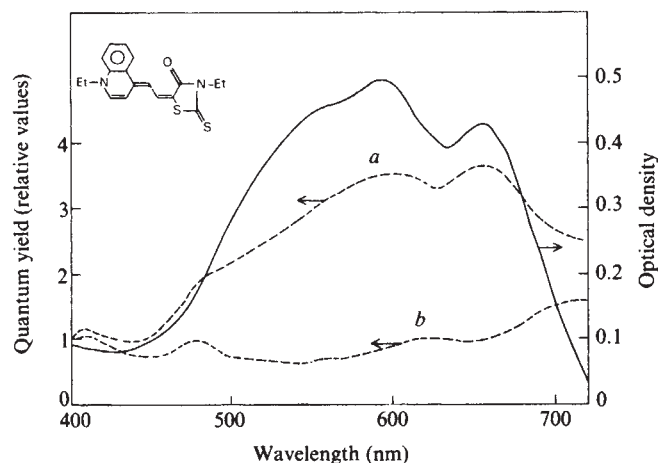


Fig. 1 Absorption spectrum and photocurrent action spectra for dye E-Ac, ~35 nm thick. *a*, Illumination through Al; *b*, illumination through Au.

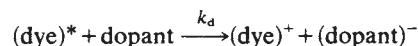
cationic form of the dye that is produced after intermolecular charge transfer in photovoltaic cells depends on the ease with which the positive charge can be delocalized. This is determined to a large extent by the ionization potential of the dye, which in turn depends on the donating ability of the donor fragment. Therefore, to assess the effect of donor strength and bridge length on photovoltaic behaviour we have screened the responses of several merocyanines containing the same acceptor group (ethyl rhodanine). Some interesting molecular structure/photovoltaic activity relationships have emerged.

Solid-state sandwich cells were prepared by the sequential vacuum evaporation and deposition of thin films of aluminium, aluminium oxide, merocyanine dye and gold onto a quartz or Pyrex substrate supplied with two indium contacts. Active areas of about 5 cm² were readily obtained. The cells were then doped with iodine by vapour diffusion. An area of 0.064 cm² was illuminated with a single-strand Crofon fibre optic. Dark and photoelectric responses were monitored throughout the preparation of the cell and the apparatus was arranged such that the vacuum was maintained at about 10⁻⁶ torr. The thickness of each layer was controlled during evaporation by means of a Kronos quartz crystal thickness monitor. The metals used were at least 99.99% pure, and the purity of the dyes (~99%), prepared at Thornton Research Centre, was checked after double recrystallization by elemental analysis, IR spectroscopy and mass spectroscopy. Redox potentials were obtained from cyclic voltammograms of CH₂Cl₂ solutions of the dyes using Pt electrodes and tetrabutyl ammonium perchlorate as supporting electrolyte against a Ag/AgCl reference electrode.

The merocyanines and data obtained are shown in Table 1. For comparison, in the solid-state cells the thickness of each dye was maintained at about 35 nm, and quantum yields (ϕ_e) based on photons absorbed by the dye (that is, correcting for transmittance of the aluminium layer) were measured at the peak of the absorption spectrum and in a range of light intensity where the photocurrent varied linearly. For donors A–E, ϕ_e followed the ease of oxidation of the dye, which correlates well with the expected trends from charge transfer behaviour, resonance theory and the Brooker deviation⁴, which give a qualitative measure of the relative donating ability of the donor residue.

Increasing the bridge length reduces the ionization potential and extends the chromophore; compare results for donors E, F and G. Unfortunately, merocyanine G-Ac became unstable during sublimation, and a thermal rearrangement to a non-coloured form, possibly a spiropyran⁵, occurred. No photovoltaic effect was observed because the compound did not absorb in the visible portion of the spectrum. The original compound, however, could be regenerated by dissolving the thin film in ethanol, suggesting that a reversible tautomerization had occurred. Merocyanine F-Ac was tested in the hope that re-

moval of the bridge between donor and acceptor groups would reduce the likelihood of tautomerization. However, no photovoltaic effect was observed, even though there was no evidence of oxetane formation. This result may be explained in terms of the excited-state lifetime of the dye. The steric hindrance between donor and acceptor groups leads to loss of coplanarity. Tredwell and Keary⁶ have shown that twisting of heterocyclic rings about the polymethine bridge to relieve steric strain enhances the rate of internal conversion to the ground state, because excited state energy can then be efficiently dissipated by intramolecular vibration and rotation. We estimate from their results that the excited state lifetime (Σk)⁻¹ for dye F-Ac is of the order of 1 ps. The charge generation process



has a first-order rate constant⁷ of about 10⁷ s⁻¹ for a dopant concentration of 10¹⁹ molecules cm⁻³. Thus, the quantum efficiency ϕ_d for excited state dissociation to charge carriers given by:

$$\phi_d = \frac{k_d[\text{dopant}]}{k_d[\text{dopant}] + \Sigma k}$$

has a value of about 0.001%, which is near the limit of detectability in our system. When combined with other energy dis-

Table 1 Oxidation potentials, ionization energies⁷ and photovoltaic quantum yields at λ_{max} for merocyanine dyes derived from ethyl rhodanine, Ac

Merocyanine	Ethyl rhodanine (Ac)					
	Relative strength of donor	Oxidation potential (V/NHE)	Ionization potential (eV)	λ_{max} (nm)	ϕ_e %	
A		Weak	1.29	5.8	420	<0.001
B		Weak	0.92*	5.4	520	0.08
C		Medium	0.98*	5.5	550	10
D		Strong	0.73*	5.2	540	14
E		Strong	0.77*	5.3	660	12
F		Strong	0.99	5.5	550	0
G		Strong	0.57*	5.1	770	Decomposed

* Irreversible, peak values recorded.

Table 2 Sunlight conversion efficiencies η_s and dependence of photocurrent I_{sc} on light intensity for Al/Al₂O₃/merocyanine, I₂/Au photovoltaic cells

Merocyanine	I_0 (mW cm ⁻²)	% Transmittance of Al	η_s %	I_{sc} , I_0 dependence
A	180*	50	$\sim 10^{-6}$	$I_{sc} \propto I_0^{0.5}$
B	63	65	2×10^{-5}	$I_{sc} \propto I_0^{0.5}$
C	52	30	0.013	$I_{sc} \propto I_0$
D	56	10	0.007	$I_{sc} \propto I_0$
E	45	27	0.18	$I_{sc} \propto I_0$
F	52	25	No response	—

I_0 is intensity of simulated AM2 sunlight incident on the Al electrode.

* Tungsten-halogen lamp.

sipitation processes, such as carrier recombination, this estimate accounts for the absence of detectable photocurrent.

The photocurrent action spectra for dye E-Ac are shown in Fig. 1. Good correspondence between action spectrum and absorption spectrum is observed for illumination through the aluminium electrode. However, an inverted behaviour is apparent when the cell is illuminated through the gold side. These results are consistent with the formation of a rectifying junction at the aluminium/dye contact. In the case of gold illumination, the dye itself acts as a filter of the strongly absorbed light, and weakly absorbed photons at the sides of the absorption peaks penetrate to the rectifying junction and make a major contribution to the photocurrent.

The dyes were tested for their photovoltaic power conversion efficiency, η_s , in simulated AM2 sunlight from a Thorn CSI lamp. η_s was calculated from the short-circuit current (I_{sc}), open-circuit voltage (V_{oc}) and fill factor (FF) at known incident light intensity (I_0) using the equation:

$$\eta_s = I_{sc} V_{oc} FF / I_0$$

and the results are shown in Table 2. Efficiencies were dependent not only on donor type but also on the relationship between I_{sc} and I_0 . The more efficient dyes, C-Ac, D-Ac and E-Ac, had linear photocurrent dependences up to and beyond the light intensities used. Taking the response for the most efficient dye, E-Ac, for a film of about 25 nm thickness, we have estimated the power conversion efficiency based on light absorbed, η'_s , defined as:

$$\eta'_s = \frac{I_{sc}}{e \int_{E_g}^{E_m} A(E) N(E) dE} \cdot \frac{e V_{oc}}{E_g} \cdot FF \times 100\%$$

$$= [\text{average quantum yield}] [\text{voltage factor}] [\text{fill factor}] \times 100\%$$

where e is the electronic charge, E_m is the high-energy limit of the solar spectrum (taken as 3.1 eV), E_g is the absorption threshold for the dye (taken as 1.7 eV), $A(E)$ is a factor which accounts for the dye absorbance and $N(E)$ is the flux density of photons incident on the dye film per wavelength interval. From estimates of the average quantum yield (0.13), voltage factor (0.42) and measured fill factor (0.39) we calculate $\eta'_s = 2.1\%$.

Further exploitation of the relationship between molecular structure and photovoltaic behaviour should lead to further improvements in the conversion efficiency.

We thank G. Ellis for preparing pure samples of the merocyanine dyes.

Received 16 July; accepted 7 November 1980.

1. Ghosh, A. K. & Feng, T. *J. appl. Phys.* **49**, 5982–5989 (1978).
2. Birks, J. B. in *Photophysics of Aromatic Molecules* Ch. 9 (Wiley, London, 1970).
3. Griffiths, J. in *Colour and Constitution of Organic Molecules* Ch. 6. (Academic, London, 1976).
4. Brooker, L. G. S. *et al. J. Am. chem. Soc.* **73**, 5332–5350 (1951).
5. Bertelson, R. C. in *Techniques of Chemistry* Vol. 3, Ch. 3 (ed. Brown, G. H.) (Wiley, London, 1971).
6. Tredwell, C. J. & Keary, C. M. *Chem. Phys.* **43**, 307–316 (1979).
7. Chamberlain, G. A. & Malpas, R. E. *JCS Faraday Discuss.* **70** (in the press).

Chemical changes during dyke metamorphism in high-grade basement terrains

Barry L. Weaver & John Tarney

Department of Geology, The University of Leicester, Leicester LE1 7RH, UK

Trace element geochemistry is an important tool in deciphering the petrogenesis of igneous, and particularly basaltic, igneous rocks, and in assigning a possible tectonic setting to these rocks^{1,2}. Studies of the trace element geochemistry of metamorphosed igneous rocks often implicitly assume element immobility during metamorphism even though recent studies^{3–7} have demonstrated the sometimes quite extreme mobility of certain trace elements, and especially of the petrogenetically important rare-earth elements (REE), during low-grade (zeolite–greenschist facies) metamorphism. The mobility of trace elements at higher grades of metamorphism is poorly known, although there is some evidence⁸ for the mobility of large ion lithophile (LIL—Rb, K, Th, Ba, Sr) elements, but not the REE, during amphibolite facies metamorphism. The REE also seem to be stable through the amphibolite to granulite facies transition^{9,10}. We have studied a suite of ultramafic to mafic dykes (the Scourie dykes) cutting retrogressed Lewisian granulites in the Assynt region, north-west Scotland¹¹ and conclude that the mobility of trace elements is not controlled solely by the degree or grade of metamorphism, but more by the availability of a fluid phase for element transfer.

The intrusion of the Scourie dolerite dykes has been dated at 2,390 Myr (ref. 12). In the Assynt region, the dyke suite may have been intruded over a time span as great as 200 Myr (ref. 13), intrusion of the early dolerites being followed by intrusion of ultramafic dykes and later, rather minor, dolerites¹¹. There is evidence for the injection and metamorphism of these dykes at depth in the crust (under pressures of 5–7 kbar) into hot (~500 °C) country rock gneisses, during the waning stages of the Inverian retrogression of Scourian granulites^{11,14–16}. The dykes are variably metamorphosed, with the earlier dykes being in general more heavily metamorphosed^{11,14}. The metamorphism of dolerite dykes from the Scourie area has been described by Sutton and Watson¹⁷, who recognized six progressive stages of metamorphism. Metamorphic conditions in Assynt (of the lower amphibolite facies) were somewhat lower than at Scourie¹¹. The metamorphism of Assynt ultramafic dykes has been detailed elsewhere¹¹.

The present study of the chemical changes associated with the metamorphism of Scourie dykes arises from the analysis of a large number of dykes from Assynt for a wide range of elements¹⁸. Amongst the trace elements, the incompatible elements are of most use in petrogenetic modelling, and it is important to assess the behaviour of these elements during metamorphism. As both fresh and variably metamorphosed dykes are present in Assynt, the behaviour of incompatible elements on metamorphism can be gauged by the normalization of the incompatible element abundances in an amphibolized dyke to those in a cogenetic fresh dyke. Ignoring possible effects due to phenocryst mineralogy, the resulting incompatible element pattern should be linear on such a plot if mobilization of trace elements has not occurred. The anomalous behaviour of an element on such a plot will, however, reflect, if phenocryst effects can be discounted, mobility of that element during metamorphism. It has been demonstrated¹⁸ that during fractionation of the cogenetic fresh Scourie dolerites, the levels of incompatible elements increase steadily, with little change in incompatible element ratios, except for Sr, which has an

approximately constant abundance due to the plagioclase fractionation which these dykes seem to have undergone. Bearing this in mind, the metamorphic effects on the chemistry of Scourie dolerites is illustrated in Fig. 1a, where the incompatible element abundances (arranged in the order given in ref. 19) in both a fresh and an amphibolized dolerite dyke have been normalized relative to a fresh dolerite. The fresh dolerite (sample 916) on this plot (Fig. 1a) displays an overall flat pattern, but with a negative Sr anomaly and a positive Ti anomaly which can be attributed to crystal fractionation effects¹⁸. On the other hand, the amphibolized dolerite (sample 689, Fig. 1a), in which pyroxene has been completely replaced by hornblende-quartz aggregates, the feldspar is altered and recrystallized, and the original texture of the rock has disappeared, displays significant relative depletion in Sr, Ba, K and Rb. The Sr anomaly can again be attributed to crystal fractionation¹⁸, but the depletion in Ba, K and Rb is a direct result of amphibolite facies metamorphism. To assess at what stage in the metamorphic process appreciable K and Rb loss occurs, the analysed dolerites have been divided into three groups:

- (1) those dykes which are fresh, with little or no replacement of pyroxene by secondary amphibole (nine samples);
- (2) those dykes in which there is complete replacement of pyroxene, but with textural preservation (four samples);
- (3) those dykes in which recrystallization of feldspar and the hornblende-quartz aggregates has occurred with destruction of the original texture (eight samples).

The average K₂O and Rb contents for the three groups are 0.82% and 18 p.p.m., 0.92% and 18 p.p.m., and 0.66% and 8 p.p.m. respectively, yielding average K/Rb ratios for each group, with 1 σ standard deviations, of 392 ± 63 , 422 ± 115 and 759 ± 356 . Clearly then, substantial loss of K and Rb (and other LIL elements), and the consequent development of high K/Rb ratios, occurs only on recrystallization of the dykes.

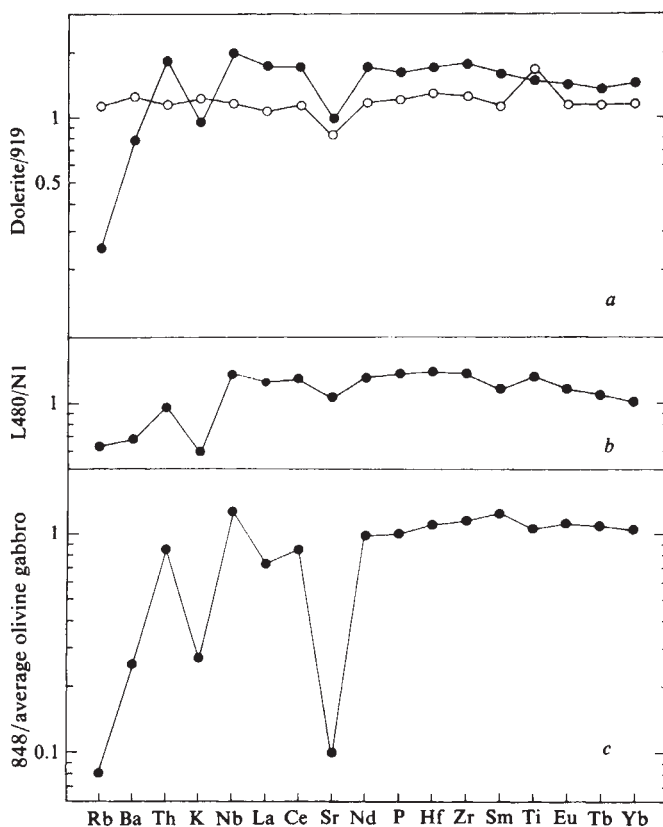


Fig. 1 Plot of incompatible element abundances in: a, fresh dolerite (○, 916) and an amphibolized dolerite (●, 689) normalized to a fresh dolerite dyke (919); b, an altered 'norite' dyke (L480) normalized to a fresh 'norite' dyke (N1); and c, an altered olivine gabbro dyke (848) normalized to the average of four fresh olivine gabbros.

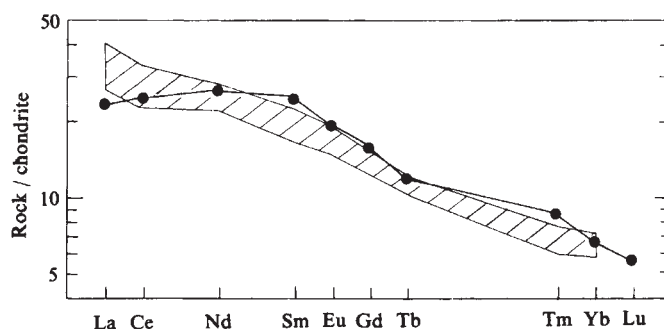


Fig. 2 Chondrite normalized plot of REE abundances in an altered olivine gabbro (●, 848) compared with the field for four fresh olivine gabbro dykes (from ref. 18). Chondrite normalizing values used are those of Nakamura²³.

Figure 1b, c investigates the metamorphism of ultramafic Scourie dykes. In Fig. 1b, sample L480 (the Loch Crocach 'norite' dyke¹¹), which is heavily amphibolized throughout its length, has been normalized to a cogenetic fresh norite dyke from Scourie (dyke N1 of ref. 15). Here the LIL elements K, Rb, Ba and, less clearly, Sr seem to have been mobilized (Fig. 1b) during metamorphism of this ultramafic dyke. However, the depletion of these elements is not particularly severe, perhaps because sample L480 shows a considerable degree of textural preservation, with fresh primary feldspar and pseudomorphs after pyroxene still obvious. On the other hand, Fig. 1c illustrates the chemical effects of metamorphism of the olivine gabbro dykes from Assynt¹¹. Sample 848 is, on the basis of most major and trace elements, clearly related to the olivine gabbro dykes¹⁸, although the original olivine-augite-orthopyroxene-plagioclase-amphibole-mica assemblage¹¹ has been totally replaced by an amphibole-chlorite-ore mineralogy, with no textural preservation. This extreme metamorphism has caused severe depletion in Sr, Ba, K and Rb in the amphibolized olivine gabbro, while Th, La and Ce are less strongly depleted. The depletion in the light REE is perhaps better illustrated in Fig. 2, where the REE abundances in 848 are compared with the range in REE patterns for four fresh olivine gabbro dykes¹⁸.

Apparently then, amphibolite facies metamorphism of Scourie dykes leads to appreciable mobilization of certain trace elements on recrystallization of the dyke. In this case, depletion in the LIL elements and the LREE is demonstrable, with mobility decreasing in the approximate order: $Rb > Ba \approx K \approx Sr \gg Th \approx La \approx Ce$, although depletion in Th, La and Ce only becomes significant on extreme amphibolization. It would also seem reasonable that the mobility of Sr will depend on the stability of feldspar: in those rocks which retain feldspar on amphibolization (the dolerite dykes and the Loch Crocach norite), Sr is not particularly mobile, while in the amphibolized olivine gabbro, which contains no relict feldspar, Sr has been severely depleted. On the other hand, the elements Nb, P, Hf, Zr, Ti and the middle- and heavy-REE are immobile for all degrees of metamorphism observed in the Scourie dykes.

The critical factor seems to be not the grade of metamorphism, but the availability of a fluid agency for element transfer. During the amphibolite-facies retrogression of the enclosing granulite-facies gneisses (which largely preceded, but overlapped, the period of dyke injection), significant chemical changes as the gneiss compositions readjusted to their new equilibrium mineral assemblages have been recorded²⁰. In this case, very considerable volumes of hydrous fluids were available to assist with element redistribution. The dykes, being intruded towards the end of this period of retrogression, were metamorphosed to a degree dependent on the availability of hydrous fluids. Thus they are metamorphosed progressively from their margins to their centres¹¹. The dyke margins, which are often sheared, provided the means of access of these hydrous fluids from below²⁰. High metamorphic temperatures alone were not sufficient to cause recrystallization, and only when the dykes had

been thoroughly metamorphosed, implying a large fluid flux, are there significant chemical changes. Conversely, low-grade metamorphism of ocean-floor basalts is frequently associated with quite large chemical changes²¹, even affecting rare-earth elements²²; however, this is frequently related to the action of hydrothermal convection cells at mid-ocean ridges in which the fluid flux is of some magnitude²¹, and high water-rock ratios are maintained for a considerable time²².

Metamorphism alone is not sufficient to initiate chemical changes; the fluid flux is a much more important factor, but one which is rather more difficult to assess. The metamorphic grade does, however, control the mineral assemblages, which determines which elements may be mobile.

B.L.W. acknowledges the receipt of an NERC research studentship. The analytical data used in this study were obtained at the University of Birmingham (X-ray fluorescence data) and the Open University (NAA data), and the use of these facilities is gratefully acknowledged.

Received 11 September; accepted 30 October 1980.

1. Pearce, J. A. & Cann, J. R. *Earth planet. Sci. Lett.* **19**, 290 (1973).
2. Winchester, J. A. & Floyd, P. A. *Earth planet. Sci. Lett.* **28**, 459 (1976).
3. Herrmann, A. G., Potts, M. J. & Knake, D. *Contr. Miner. Petrol.* **44** (1974).
4. Humphris, S. E., Morrison, M. A. & Thompson, R. N. *Chem. Geol.* **23**, 125 (1978).
5. Ludden, J. N. & Thompson, G. *Earth planet. Sci. Lett.* **43**, 85 (1979).
6. Hellman, P. L., Smith, R. E. & Henderson, P. *Contr. Miner. Petrol.* **71**, 23 (1979).
7. Nesbitt, H. W. *Nature* **279**, 206 (1979).
8. Muecke, G. K., Pride, C. & Sarkar, P. *Phys. Chem. Earth* **11**, 449 (1979).
9. Green, T. H., Brunfelt, A. O. & Heier, K. S. *Geochim. cosmochim. Acta* **36**, 241 (1972).
10. Compton, P. *Contr. Miner. Petrol.* **66**, 283 (1978).
11. Tarney, J. in *The Early Precambrian of Scotland and Related Rocks of Greenland* (eds Park, R. G. & Tarney, J.) 105 (University of Keele, 1973).
12. Chapman, H. J. *Nature* **277**, 642 (1979).
13. Evans, C. R. & Tarney, J. *Nature* **204**, 638 (1964).
14. Tarney, J. *Nature* **199**, 672 (1963).
15. O'Hara, M. J. *Miner. Mag.* **32**, 848 (1961).
16. O'Hara, M. J. *J. geol. Soc. Lond.* **134**, 185 (1977).
17. Sutton, J. & Watson, J. V. *Geol. Mag.* **88**, 25 (1951).
18. Weaver, B. L. & Tarney, J. (in preparation).
19. Wood, D. A., Joron, J.-L., Treuil, M., Norry, M. J. & Tarney, J. *Contr. Miner. Petrol.* **70**, 319 (1979).
20. Beach, A. & Tarney, J. *Precamb. Res.* **7**, 325 (1978).
21. Donnelly, T. W., Thompson, G. & Robinson, P. T. *Am. geophys. Un. Maurice Ewing Ser.* **2**, 369 (1979).
22. Menzies, M., Seyfried, W. & Blanchard, D. *Nature* **282**, 398 (1979).
23. Nakamura, N. *Geochim. cosmochim. Acta* **38**, 757 (1974).

An unusual bed of giant pumice in Mexico

B. J. Clough, J. V. Wright*† & G. P. L. Walker*

Department of Geology, Imperial College, London SW7 2BP, UK

We report here that a remarkable bed of giant-sized pumice blocks occurs within an old caldera of the major late Quaternary rhyolitic volcano of Sierra La Primavera, Mexico (Fig. 1a). The bed lies within a 100-m thick sedimentary sequence distributed over a near-circular area 10 km across. The margin of this circular area closely parallels an incomplete ring of porphyritic comendite lava domes (Fig. 1b), some of which may be shallow intrusions emplaced into the sediments. The vents of these domes are believed to lie on a ring fault (Fig. 1b). The sedimentary sequence, now gently updomed (Fig. 1c), is believed to have accumulated in a caldera lake, the shoreline of which lay up to 1 km outside the ring fault.

The lake sediments are floored by an ignimbrite (Rio Caliente Ignimbrite) produced by the biggest explosive eruption of the volcano^{1,2}; many of the deep gullies within the badland terrain, which characterize the area of lake deposits, cut down into the floor. The lake deposits have a maximum exposed thickness of ~100 m, and rest on top of the ignimbrite which was eroded before their accumulation. Locally, the non-welded top of the

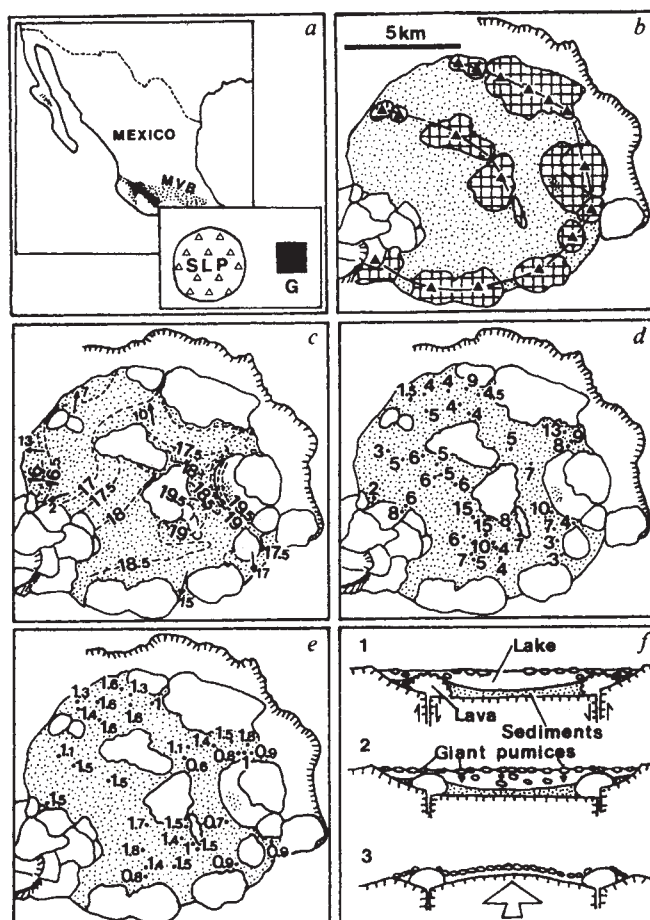


Fig. 1 The giant pumice block bed and lake sediments, and their relationships to the young rhyolite lavas of La Primavera volcano. *a*, Index map of study area: MVB is the Mexican Volcanic Belt which is a locus of Quaternary volcanism in Mexico; G is Guadalajara; SLP is the Sierra La Primavera. *b*, Distribution of the lake sediments and rhyolite lavas: stipple is lake sediments; cross-hatch is porphyritic comendite lavas lying on an 'inner-ring' with a transverse zone; triangles represent inferred vent positions; unornamented lavas are aphyric comendite lavas lying on an 'outer-ring' which is not fully shown or discussed here; hatched line is a low escarpment, possibly an embryo caldera rim. *c*, Altitude contours of the top of the giant pumice block bed in hundreds of metres above sea level. Heights were determined in the field using an altimeter. Dip of lake sediments is also shown (arrows). *d*, Thickness of giant pumice block bed in metres. *e*, Median diameter of giant pumice blocks in metres; 65–20 blocks were measured at each outcrop. *f*, Three stages in the envisaged formation of the giant pumice block bed.

ignimbrite (at least 15 m) was stripped off and box canyons were incised into the incipiently welded zone. Some of these canyons are infilled with cross-stratified and pumice-rich fluvatile deposits.

The lake deposits are planar-laminated white vitric ashes and pumice layers. Some ash beds contain diatoms; discrete beds of diatomite also occur. The sediments commonly have current-ripple laminae and load casts indicative of deposition under water. Diatom assemblages indicate that the water was shallow (G. H. Evans, personal communication) and less saline than present-day lakes in this area (J. P. Bradbury, personal communication).

The most distinctive unit in the lake-sediment succession is the giant pumice block bed (Fig. 2a), which is 1.5–15 m thick and can be traced over practically the entire former lake area (Fig. 1d). It has an extreme bimodal size distribution, the coarse mode consisting of giant blocks of light grey porphyritic pumice averaging 1.3 m in size (Fig. 1e), commonly grain supported, with the finer mode consisting of fine white ash occurring

* Present addresses: Department of Geology, University of Puerto Rico, Mayaguez, Puerto Rico 00708 (J. V. W.) and Department of Geology, University of Auckland, New Zealand (G. P. L. W.).
† To whom correspondence should be addressed.

between the large blocks. The giant pumice blocks (Fig. 2) range in diameter from 0.2 to generally less than 6 m and are thus quite well sorted for their size; the maximum diameter measured of any block was 8.5 m. Locally, the giant pumice block bed is underlain by a thick accumulation of smaller-sized pumice. Near the northern edge of the lake area, the bed occurs as two or three beds separated by stratified reworked ash and pumice. The giant pumice blocks in the upper two beds are more matrix supported than is usual for this deposit, and may be reworked.

The ash matrix in the giant pumice bed is laminated, and the laminations are disturbed and contorted around the pumice (Figs 2a, 3); this is interpreted as gentle settling of the pumice blocks into waterlogged ash. However, the laminations are also disturbed above the pumice blocks, which is interpreted as a compactional feature. The pumice forms are oblate spheres and poorly rounded blocks (Figs 2b, 3). Some have a glassy, poorly vesicular outer sheath about 1 cm thick, but this is discontinuous. Many of the blocks have a crude prismatic jointing normal to their margins, most clearly developed in the outer part of the blocks; occasionally this jointing is very well developed (Fig. 2b). These features indicate that the giant pumice blocks have been chilled. A small amount of abrasion evidently took place before deposition.

Features such as relative uniformity of pumice size, relative uniformity in thickness (Fig. 1d), maximum pumice sizes (Fig. 1e) of the bed over practically the entire former lake area, and the lack of bomb sags make the giant pumice block bed sufficiently unique to demand an unusual mechanism of formation. Pyroclastic processes are inadequate to account for these features. The following mechanism (Fig. 1f), consistent with all the observed field relationships, envisages porphyritic comendite lava extrusions forming on the floor of a lake. It is common for the carapace of a silicic lava extrusion to be pumiceous, and

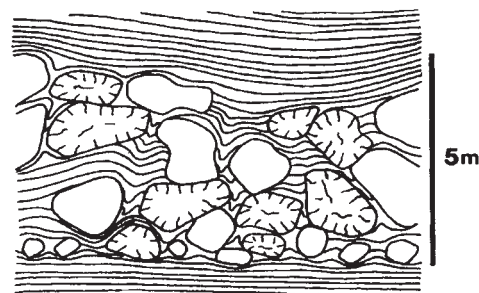


Fig. 3 Field sketch showing disturbed and contorted lake sediments with blocks of giant pumice. Some blocks show cooling joints normal to their margins.

in this case pumice blocks became detached from the carapace and floated to the lake surface. Eventually, the entire surface was covered with giant blocks of floating pumice, and these blocks became evenly distributed by jostling against one another. Size-sorting occurred as smaller pumice fragments rapidly sank close to the extrusions. Eventually, however, the largest pumice blocks became waterlogged and sank to generate the coarse mode of the bed. In places the bed mantles porphyritic lava, and here rests on a thick apron of less coarse pumice which sank earlier (if, indeed, it floated at all).

Various lines of evidence indicate that the porphyritic lavas were emplaced in water or wet lake sediments. These include, (1) an unusually thick carapace of glassy rocks which is invariably present, (2) particularly well developed jointing (in places the rocks are extensively brecciated), (3) anomalously close spacing of microfractures and a resulting very pervasive perlitic alteration, and (4) an absence of spherulites and lack of post-eruption crystallization in general. In contrast, the aphyric comendite lavas of the volcano (Fig. 1b) are thought to have been erupted 'dry'. They have a carapace of fresh or slightly perlitized obsidian interbanded with stony and spherulitic rhyolite, and close-spaced foliation planes which are steeply inclined and intensely contorted.

The origin of the lake depression in which the sediments accumulated is not immediately apparent, because subsequent uplift of the central part of the volcano masks the original relationships (Fig. 1c). Geological relationships, however, suggest that the lake occupied a caldera. The sediments extend up to 1 km outside the limit of the rhyolitic ring, which conforms with observations that the topographic rim of a caldera commonly lies outside the ring fault³ (as marked by lava extrusions or explosive vents). There is, in fact, a low escarpment 1–2 km outside the lava ring on the northern and eastern side (Fig. 1), but this could be a second outer embryo caldera of a nested pair. Resurgent updoming⁴ has caused an inversion of topography so that the lake deposits now stand well above any possible shoreline. The updoming took place relatively late in the history of the volcano and did not follow closely on the climatic ignimbrite eruption. At La Primavera this could indicate the arrival of a new pluton beneath the volcano. If so, the apparently youthful age of the updoming indicates that possible future activity of the volcano should be considered seriously². Numerous hot springs within the volcano point to the existence of a hydrothermal system driven by heat from a hot rock or magma body.

We thank NERC for funding field work in 1976 and 1977, and the Director (Ing. Diego A. Cordoba) of the Instituto de Geología, UNAM, for logistic support. B.J.C. and J.V.W. held NERC research studentships during this work.

Received 28 July; accepted 28 October 1980.

1. Wright, J. V. thesis, Univ. London (1979).
2. Walker, G. P. L., Wright, J. V., Clough, B. J. & Booth, B. *Geol. Rundsch.* (in the press).
3. Lipman, R. W. *Geol. Soc. Am. Bull.* **87**, 1397 (1976).
4. Smith, R. L. & Bailey, R. A. *Geol. Soc. Am. Mem.* **116**, 613 (1968).

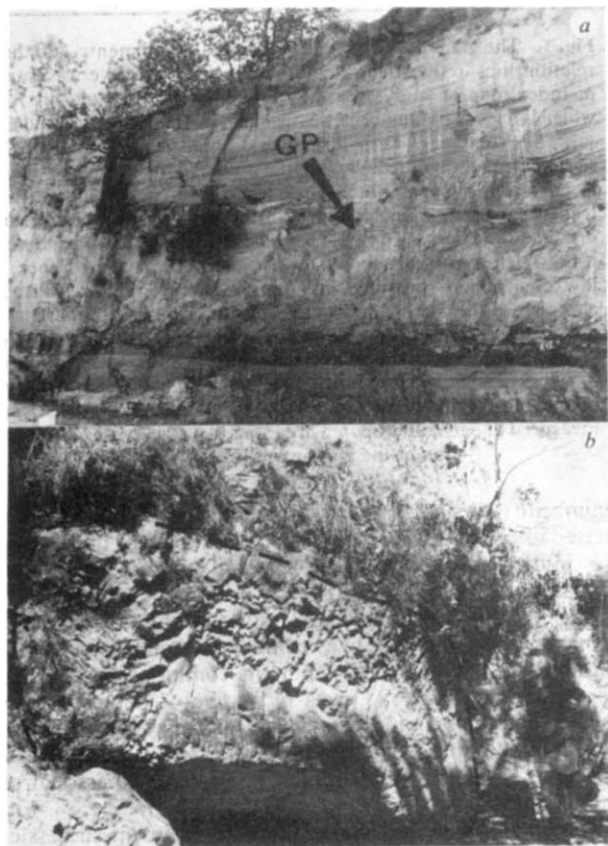


Fig. 2 a, Giant pumice block bed (GP), at this location approximately 5 m thick, and laminated lake sediments which are disturbed and contorted. b, Giant pumice block; this example has a largest diameter of ~6 m, and also has very well developed prismatic jointing normal to its margins.

Microfossil-like objects from the Archaean of Greenland: a cautionary note

D. Bridgwater*¶, J. H. Allaart*, J. W. Schopf†, C. Klein†‡, M. R. Walter†**, E. S. Barghoorn‡, P. Strother‡, A. H. Knoll§ & B. E. Gorman||

* Geological Survey of Greenland Øster Voldgade 10, DK-1350 Copenhagen K, Denmark

† Precambrian Paleobiology Research Group, Department of Earth and Space Science, University of California, Los Angeles, California 91124

‡ Department of Biology, Harvard University, Cambridge, Massachusetts 02138

§ Department of Geology, Oberlin College, Oberlin, Ohio 44074

|| Department of Geology, The University of Western Ontario, London, Canada N6A 5B7

Recent reports¹⁻⁴ have described "yeast-like microfossils" (*Isuasphaera isua* Pflug²) in 3,800-Myr old⁵ metaquartzites from the Isua supracrustal belt of south-west Greenland. A biogenic interpretation of these objects is inconsistent with the tectonic history of the Isua region⁶⁻⁸, with the petrology of the metaquartzites, and with the morphology of the microstructures themselves. The putative microfossils are indistinguishable from limonite-stained fluid inclusions: microstructures which are demonstrably inorganic and post-depositional in origin. As such, we contend here that these objects should not be regarded as evidence of early Archaean life forms.

The geological history of the Isua supracrustal belt is summarized in Table 1. Several episodes of deformation, geochemical alteration and amphibolite-facies metamorphism have been recognized, in sharp contrast to slightly younger Archaean strata from which stromatolites^{9,10} and apparently *bona fide* microfossils¹¹⁻¹³ have been reported.

Evidence of penetrative deformation is seen throughout the Isua succession where all lithologies record high strains. Few primary sedimentary and igneous structures are preserved apart from the gross lithological layering which allows a rudimentary stratigraphy to be described. Even in fold hinges or inclusions of competent material in a ductile matrix where strain is comparatively low a strong linear fabric is developed and primary features are only recognizable on surfaces normal to the maximum extension. Original discordant structures, such as the contacts between the supracrustal rocks and the intrusive Amitsoq gneisses have been rotated to near-parallel. The absence of strain markers in the main quartzite horizon (from which the fossil-like microstructures were found), obscures evidence of deformation. Nonetheless, thin layers of magnetite and cumingtonite in the quartzites exhibit extension fabrics similar to those in neighbouring ironstones. There is no evidence that the quartzite horizon escaped the episode(s) of high deformation.

Large-scale geochemical alteration is inferred from rocks interpreted as volcanic in origin. Basic rocks record massive remobilization of alkalis, silica and CO₂ (ref. 14), whereas acid rocks are strongly enriched in K₂O (refs 6, 7) or carbonate minerals. Ultrabasic units (dunites and peridotites) are commonly transformed into schistose assemblages of talc, Mg-amphibole and carbonates. Sedimentary carbonate units display high initial Sr-isotope ratios (see W. Compston in ref. 15) attributed to massive metasomatic changes in constituents.

At least five regional metamorphic events occurred between 3,700 and 1,600 Myr (Table 1). The supracrustal rocks reached amphibolite-facies metamorphism twice: once associated with the emplacement of the Amitsoq gneisses (~3,700 Myr), and again following the intrusion of the Ameralik dykes (before 3,000 Myr). Efforts to quantify temperatures and pressures attending these metamorphic events have been hampered by the

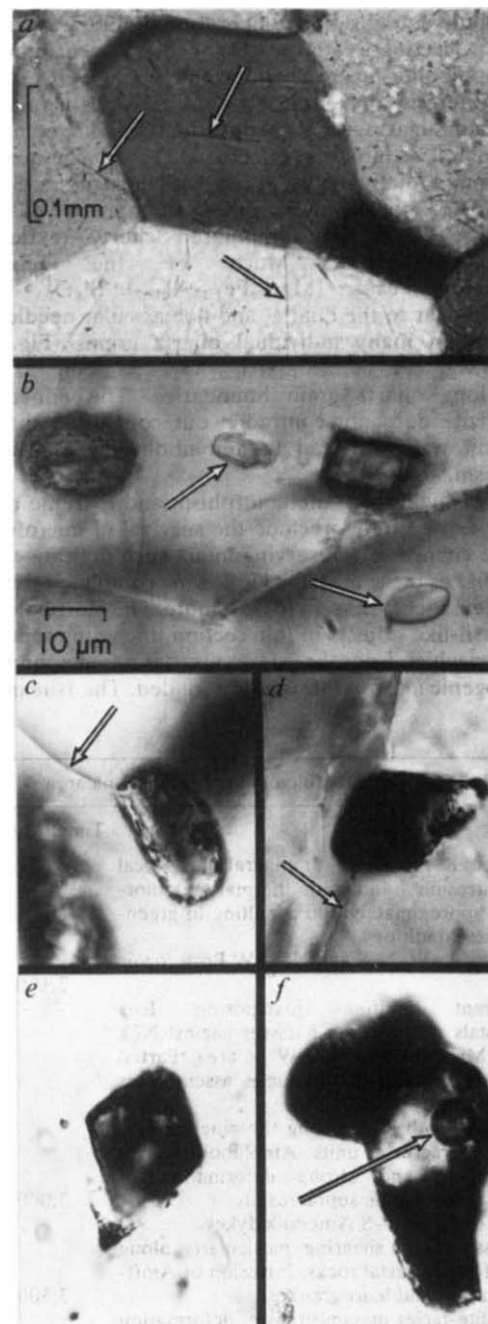


Fig. 1 Optical photomicrographs showing recrystallized, equigranular quartz grains (in plane polarized light; *a*) and non-biogenic, apparently limonite-stained microfossil-like objects (in transmitted white light; *b-f*) in petrographic thin sections of greenish-white quartzite from the 3,800 Myr Isua supracrustal belt of Greenland; *a*, arrows point to fine actinolite needles; *b*, arrows point to clear, ellipsoidal, fluid inclusions; *c, d*, arrows point to limonite-stained quartz grain boundaries (also visible in *b*); *e*, arrow points to gas bubble within an elongate, mineral-stained inclusion. *b-f* (scale as in *b*) are from thin section no. 60464, Paleobotanical Collections, Harvard University, Cambridge, Massachusetts.

¶ Present address: The Geological Museum, Øster Voldgade 5, DK-1350 Copenhagen.

Permanent address: Department of Geology, Indiana University, Bloomington, Indiana 47405.

** Present address: Bureau of Mineral Resources, Geology and Geophysics, PO Box 378, Canberra, ACT, Australia 2601.

polymetamorphic nature of the rocks and their common retrogression to chlorite-bearing greenschist facies assemblages. High-grade mineral assemblages recognized from different rock types are listed in Table 2. With specific reference to the pelitic units, the assemblage biotite + garnet + quartz + staurolite and the presence of kyanite¹⁶ are consistent with temperatures of 500–600 °C and pressures of 200–500 MPa. Independent evidence of the temperatures of metamorphism is given by the oxygen isotope fractionation in quartz–magnetite pairs of the main quartzite horizon¹⁷. These ratios indicate a mean (and likely minimum) equilibration temperature of 390 °C, which we interpret as reflecting a late (post-3,000 Myr) event following regional amphibolite-facies metamorphism.

The 'fossiliferous' rock, specimen no. 2377, is a relatively coarse-grained, sugary, greenish-white quartzite. It is composed of quartz (95%), actinolite (4%) and talc (1%). The quartz is equigranular and totally recrystallized, with most grains clustering in the 0.2–0.3 mm size class. Neither the presence of "chalcedony" in the rock^{1,3,4}, nor its supposed "cherty" texture^{1,2,4}, could be confirmed. Much of the actinolite ($\text{Ca}_{1.44}\text{Na}_{0.04}\text{Mn}_{0.07}\text{Fe}_{0.45}$) ($\text{Mg}_{3.79}\text{Fe}_{1.13}\text{Al}_{0.07}$) Si_8O_{23} is of a grain size similar to the quartz, and fine acicular needles can be found within many individual quartz grains (Fig. 1a). The talc ($\text{Mg}_{5.19}\text{Fe}_{0.87}$) ($\text{Si}_{7.90}\text{Al}_{0.07}$) O_{22} is seen as fine "beards" along quartz grain boundaries. The mineralogy of the quartzite does not contradict our conclusion that the quartzite unit was subjected to amphibolite-facies regional metamorphism.

Evidence of high grade metamorphism and extreme deformation does not entirely preclude the survival of microfossils, although the chances of preserving intact such delicate carbonaceous bodies as those described following complete recrystallization of their host rock seem to be remote. The morphology of the microfossil-like objects in thin section (including specimen no. 2377 from which *Isuasphaera* was first described²) convinces us that a biogenic interpretation is unfounded. The Isua micro-

Table 2 Amphibolite-facies mineral assemblages* in the Isua supracrustal rocks

Rock type	Assemblage
Ironstones (and Mg-rich analogues)	Quartz, magnetite, grunerite/cummingtonite
Basic volcanic rocks	Hornblende, plagioclase (andesine–labradorite), quartz, magnetite, sphene (diopside)
Mg-rich and ultrabasic volcanic rocks	Talc, anthophyllite, carbonate minerals (quartz, plagioclase)
Carbonate rocks and calc-silicates	Diopside, anthophyllite, Fe-carbonate, calcite
Pelites	Biotite, quartz, garnet, magnetite (staurolite, kyanite)

* Minerals in parentheses are only sporadically preserved.

fossil-like objects are reported to "consist predominantly of carbon... characterized by a high degree of bituminization"¹ preserved "partly in a charred condition, partly close to a final stage of graphitization"⁴; they have shapes ranging from ellipsoidal to elongate with "individuals... found in the assemblages which resemble a biscuit, a dumb-bell, or a pair of tears"¹. The objects range in size from 10 to 40 µm (with "mature cells" said to occur towards the upper end of the size range³), and they are described as being "hollow" with their interiors "partly filled with organic matter or fine-grained pyrite" which is "dark brownish" in colour^{1,3}. They are also reported to exhibit structures interpreted as "cell walls", "cell vacuoles", "gas vacuoles", "multilaminate sheaths", "budding structures", "bud scars", "cross walls", "remnants of protoplasm" and evidence of "filamental growth"^{1,3}. By comparison with illustrations of dried, carbol fuchsin-stained yeast cells, and on the basis of the features noted above, these objects have been interpreted as representing carbonaceous cellularly intact remnants of "yeast-like organisms", possibly of "an evolutionary level which is far below that of yeasts of today" but which "may represent a half-way line between a microsphere-like protobiont and subsequent evolution"^{1,3}.

Our thin sections contain microstructures which conform in size, shape and all other optically detectable properties to the putative microfossils previously described, leaving no doubt that our material is identical to that previously interpreted as biogenic. We find, however, that the populations of these objects span a decidedly broader size-range than that previously reported: measurements of 250 such objects (in three thin sections) show that they range in length between 2 and 115 µm, with a mean length of ~23 µm (and a variance, s^2 , of 38.2).

Two principal categories of fossil-like objects occur in the thin sections: (1) clear, spherical to ellipsoidal structures (Fig. 1b) occur only within quartz grains (rather than at grain boundaries); these structures are comparable to fluid inclusions: inorganic, metamorphically produced micro-structures that are of well known and relatively common occurrence in other, similarly altered metasediments¹⁸; (2) yellowish to reddish-brown (apparently limonite-stained) structures that exhibit a continuum of shape from spheroidal to ellipsoidal to sub-angular to completely euhedral (Fig. 1b–f).

With the apparent exception of the occurrence of "multilaminate sheaths" (of which we find no evidence) members of this second category are identical to forms described as *Isuasphaera*^{1–4}. However, the population includes rhombohedral (Fig. 1e) and obviously crystalline individuals (see also ref. 1 Fig. 3, parts 2, 4, 11, 12) which do not exhibit the pattern of morphological consistency characteristic of fossilized micro-organisms¹⁹. Furthermore the objects show a marked concentration at quartz grain boundaries (Fig. 1c, d), a feature commonly observed in fluid inclusions in recrystallized granites²⁰

Table 1 Simplified table of events in the Isua area

Events	Time (Myr BP)
10. Resetting of K–Ar and Rb–Sr mineral ages ²⁵ local granite intrusion ²⁶ and static thermal metamorphism to approximately 300°, faulting in greenschist facies conditions.	1,600
9. Intrusion of E–W, N–S and NE–SW Proterozoic dykes.	2,100–
8. Transcurrent faulting juxtaposing Isua supracrustals and Amisoq gneisses against Nuk (~3,000 Myr) gneisses in NW of area. Partial retrogression of amphibolite-facies assemblages in supracrustals.	
7. Deformation with local strong shearing concentrated in supracrustal units. Amphibolite-facies metamorphism and strong deformation of Ameralik dykes within supracrustals.	3,000–
6. Intrusion of E–W, N–S Ameralik dykes.	
5. Retrogression and shearing particularly along contact of supracrustal rocks. Intrusion of Amisoq pegmatites and leucogranites.	3,500–
4. Amphibolite-facies metamorphism, deformation to produce major stretching fabric in supracrustals.	
3. Intrusion of Amisoq tonalitic and granodioritic gneisses.	3,700–
2. Deformation of supracrustals. Fabric locally preserved, mineral assemblages commonly retrogressed during later events.	
1. Deposition of Isua sediments, volcanism, post-depositional hydrothermal activity.	3,800–

Ages quoted are rounded to the nearest 100 Myr. Based on published and unpublished Rb–Sr, Sm–Nd, Pb–Pb whole-rock, Pb–Pb, U/Pb, Rb–Sr and K–Ar mineral determinations. See refs 5–8 for original refs.

and not likely to be the result of a primary sedimentological distribution. The reported "budding", which has previously been attributed to a biological origin, we interpret as a 'necking-off' process commonly occurring during recrystallization of multiphase inclusions²¹, while the reported "gas vacuoles" are bubbles trapped within the objects during their formation and recrystallization¹⁸. Other suggested biological features (bud scars, cross walls, and so on) are more consistent with an inorganic origin, for example, the staining of the surfaces of fluid inclusions and negative crystals by secondarily mobilized limonitic iron oxides which were also deposited along grain boundaries. Many of the quartz grain boundaries are, in fact, stained with limonite and are indistinguishable in colour and texture from the coating that outlines the fossil-like objects (see ref. 4, plate 2, Figs 2, 5, 6).

Thus consideration of the geological, petrological and morphological evidence leads us to conclude that the fossil-like microstructures (*Isuasphaera isua*) described from the 3,800-Myr old Isua supracrustal rocks are in fact non-biogenic artefacts of inorganic, post-depositional origin. Their reported "yeast-like" morphology and fine structure are inconsistent with the tectonic history of the rocks in which they occur and are at variance with the remainder of the Precambrian fossil record as now known²². The occurrence of hydrocarbons within these microstructures³, if confirmed, would be equally consistent with both a biological and non-biological origin of these compounds. We believe that the microstructures are non-biogenic, and while graphitic carbonaceous matter occurs in the Isua metasediments^{4,23,24} it remains to be established whether this is biogenic or abiogenic in nature and to what extent it may be of postdepositional rather than solely syngenetic origin.

We thank M. Schidlowski for providing sample no. 2377 and P. Appel and C. Walters for additional material, and W. S. Fyfe, G. Perry, R. Dymek, R. Kerrich and J. D. Meloeche for information and discussion. Mapping at Isua (D.B. and J.H.A.) is part of the programme of the Geological Survey of Greenland. Field work was supported by NATO research grant no. 949 and by a NSERC (Canada) grant to W. S. Fyfe (B.E.G.). We thank other members of the NATO supported research group⁸ for discussion. Laboratory work was supported by NSF grants 77-22518 and 79-21777 and by NASA grants NGR 05-007-407 and NSG 7489 (to J.W.S.); by NASA grant NGL 22-007-067 and by NSF grant EAR 78-24237 (to E.S.B.) by NSF grant EAR 76-11740 (to C.K.).

Received 2 May, accepted 10 November 1980.

- Pflug, H. D. *Naturwissenschaften* **65**, 611-615 (1978).
- Pflug, H. D. *Oberherr. Naturw.* **44**, 131-145 (1978).
- Pflug, H. D. & Jaeschke-Boyer, H. *Nature* **280**, 483-486 (1979).
- Pflug, H. D., Jaeschke-Boyer, H. & Sattler, E. L. *Microsc. Acta* **82**, 255-266 (1979).
- Moorbath, S., O'Nions, R. K. & Pankhurst, R. J. *Nature* **245**, 138-139 (1973).
- Bridgwater, D., Keto, L., McGregor, V. R. & Myers, J. S. in *Geology of Greenland* (eds Escher, A. & Watt, W. S.) 18-75 (Geological Survey of Greenland, 1976).
- Allaart, J. H. in *The Early History of the Earth* (ed. Windley, B. F.) 177-189 (Wiley, New York, 1976).
- Bridgwater, D. *et al. Rapp. Grønland geol. Unders.* **95**, 66-71 (1979).
- Walter, M. R., Buick, R. & Dunlop, J. S. R. *Nature* **284**, 443-445 (1980).
- Lowe, D. *Nature* **284**, 441-443 (1980).
- Schopf, J. W. & Barghoorn, E. S. *Science* **156**, 508-512 (1967).
- Muir, M. D. & Grant, P. R. in *The Early History of the Earth* (ed. Windley, B. F.) 595-604 (Wiley, New York, 1976).
- Knoll, A. H. & Barghoorn, E. S. *Science* **198**, 396-398 (1977).
- Gill, R. C. O., Bridgwater, D. & Allaart, J. H. *Spec. Publ. geol. Soc. Aust.* (in the press).
- Apple, P. W. U. *Precamb. Res.* **11**, 83 (1980).
- Boak, J. L. & Dymek, R. F. *Geol. Soc. Am. Abstr.* (in the press).
- Perry, E. C., Ahmad, S. N. & Swulius, T. M. *J. Geol.* **86**, 223-239 (1978).
- Deicha, G. *Les Lacunes des Cristaux et leurs Inclusions Fluides. Signification dans la Genèse des Gîtes Minéraux et des Roches* (Masson et Cie, Paris, 1955).
- Schopf, J. W. *Origins of Life* **7**, 19-36 (1976).
- Wilkins, R. W. T. & Barkas, J. P. *Contr. Miner. Petrol.* **65**, 293-299 (1978).
- Roedder, E. *Fluid Inclusion Res. Proc. Conf.* **4** (1968).
- Schopf, J. W. *A. Rev. Earth planet. Sci.* **3**, 213-249 (1975).
- Nagy, B., Zumberge, J. E. & Nagy, L. A. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1206-1209 (1975).
- Schidlowski, M., Appel, P. W. U., Eichmann, R. & Junge, C. E. *Geochim. cosmochim. Acta* **43**, 189-199 (1979).
- Pankhurst, R. J., Moorbath, S., Rex, D. C. & Turner, G. *Earth planet. Sci. Lett.* **20**, 157-170 (1973).
- Kalsbeek, F., Bridgwater, D. & Boak, J. *Rapp. Grønlands geol. Unders.* **100**, 73-75 (1980).

Amino acids and hydrocarbons ~3,800-Myr old in the Isua Rocks, southwestern Greenland

Bartholomew Nagy*, Michael H. Engel*‡,
John E. Zumberge*§, Hiroshi Ogino*
& Sai Y. Chang†||

* Laboratory of Organic Geochemistry, Department of Geosciences, The University of Arizona, Tucson, Arizona 85721

† Department of Pharmacology, The University of Arizona, Tucson, Arizona 85721

The Isua supracrustal belt consists of metamorphic rocks, including metamorphosed sediments, and are the oldest known rocks on Earth. It has been postulated^{1,2} that hydrocarbons recently detected in these rocks are remnants of organisms that lived ~3,800 Myr ago. This popular interpretation is inconsistent with the high-temperature history of the Isua rocks. Various types of hydrocarbons were liberated from these rocks after repeated solvent extraction by matrix dissolution or pyrolysis. However, the Isua rocks have been metamorphosed to the upper greenschist or amphibolite facies (400-600 °C); the metamorphic episodes lasted >10⁶ yr. Consequently, it is very important that the origins of the key organic constituents be established. In the study reported here both hydrocarbons and amino acids were detected in Isua rock samples. In addition to the common biological amino acids, geologically unstable amino acids and biologically uncommon ones were detected (such as sarcosine). The extent of amino acid racemization suggests the apparent continuous diffusion of biochemicals into the Isua rocks from encrusting lichens since the end of the last ice age. Also, the cold-temperature history (which retards racemization) suggests that the amino acids may be modern to a few tens of thousands of years old. *n*-Alkanes lacked odd/even carbon chain preference which may indicate some antiquity. However, the hydrocarbons also resemble a recent petroleum distillate fraction. Laboratory experiments, supported by kinetic and thermodynamic calculations, show that amino acids and aromatic and saturated aliphatic hydrocarbons, including pristane, could not have survived the known metamorphic history of the Isua rocks.

Samples from the Isua banded iron formation (~150 km north-east of Godthåb) were analysed. Sample 155782 was an interior portion of the banded ironstone (~50 g) that contained a limonite-filled microcrack. Samples 3015, 3016, 3022 and 3028 (~50 g each) had weathered surfaces. Porosity, air and water permeabilities of samples 3015 and 3016 were 2.3%, 1.0×10⁻² mdarcies and 1.2×10⁻⁶ mdarcies; and 2.2%, 4.6×10⁻¹ mdarcies and 1.3×10⁻¹ mdarcies, respectively³. Isua sample 3015 was less extensively weathered than Isua 3016 and thus had a lower water permeability.

To minimize laboratory contamination, all glassware was acid cleaned, solvents and acids were redistilled from reagent or spectral grade, water was triple distilled in glass and so on^{4,5}. The rock surfaces were drilled off and the samples pulverized. Excessive ball milling (>20 h) was avoided because it caused some racemization of amino acids. The pulverized samples and corresponding blanks were refluxed with water for 8 h. The filtrate residues were hydrolysed for 24 h at 100 °C in 6 M HCl. The samples were then desalted by ion exchange chromatography^{4,5} and the *N*-PPF-(+)-2-butyl amino acid diastereomers were prepared^{4,5} and analysed for their D/L amino acid ratios by gas chromatography. Amino acids in the water extract of sample 3028 were analysed by combined gas chromatography-chemical ionization mass spectrometry (GC-CIMS). Part of the water extract of Isua sample 3016 was analysed by an amino acid analyser.

Present addresses: ‡Carnegie Institution of Washington, Geophysical Laboratory, 2801 Upton St. NW, Washington, DC 20008; §Cities Service Co., Box 50408, Tulsa, Oklahoma 74150; ||McNeil Laboratories, 500 Office Center Drive, Fort Washington, Pennsylvania 19034.

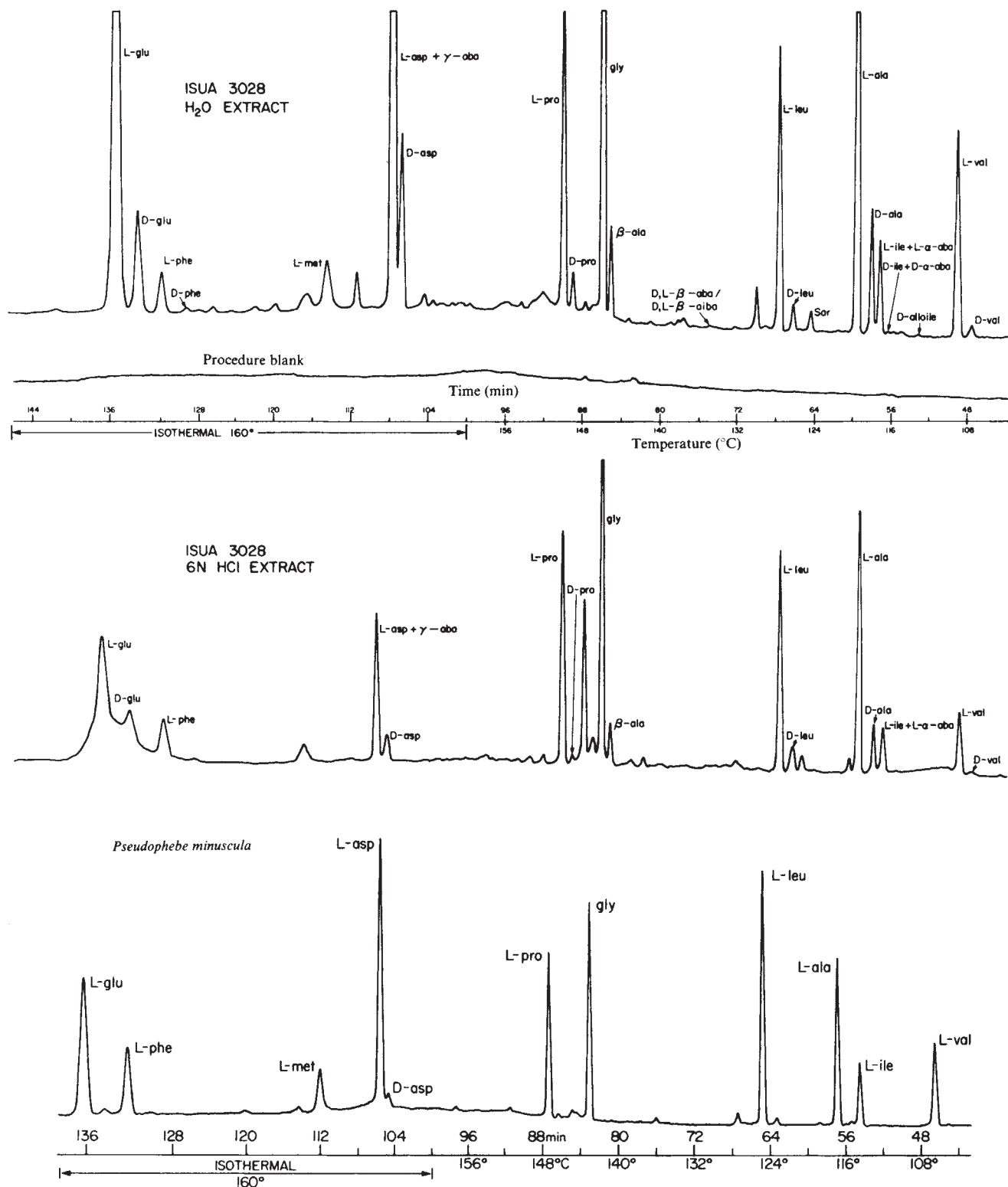


Fig. 1 Gas chromatograms of the water extract, procedure blank and HCl acid extract of Isua sample 3028 and of the 6M HCl extract of the lichen *Pseudophebe minuscula*. A Perkin-Elmer model F-11 gas chromatograph coupled to an Infotronics CRS-208 integrator was used with a Carbowax-20M 61m $\times$ 0.5 mm i.d. WCOT nickel capillary column. The He flow rate was 11.5 ml min⁻¹. The inlet temperature was 150 °C. The oven programme was 80 °C isothermal for 20 min; then 80 °C to 160 °C at 1 °C min⁻¹. The programmes were finished isothermal at 160 °C. In addition to co-injection of amino acid standards and retention times, all amino acid identifications in the water extract of sample 3028 were confirmed by GC-CIMS. A Finnigan 9500 gas chromatograph coupled to a Finnigan 3300 mass spectrometer (quadrupole) equipped with a 6110 series data system was used. CH₄ served as both carrier gas and reactant for chemical ionization. A Carbowax-20M 46 m $\times$ 0.5 mm i.d. SCOT stainless-steel capillary column was used for GC-CIMS. The carrier gas flow rate was $\sim$ 8.5 ml min⁻¹. The oven programme was 90 °C to 150 °C at 2 °C min⁻¹. The programme was finished isothermal at 150 °C. The ionization chamber temperature and pressure were 150 °C and 1.0 Torr, respectively. The emission current was 1.0 mA. 150 eV was used to generate the methane ions. Valine, val; alloisoleucine, alloile; α -amino-*n*-butyric acid, α -aba; isoleucine, ile; alanine, ala; sarcosine, sar; leucine, leu; β -amino-*n*-butyric acid, β -aba; β -aminoisobutyric acid, β -aiba; β -alanine, β -ala; glycine, gly; proline, pro; γ -amino-*n*-butyric acid, γ -aba; aspartic acid, asp; methionine, met; phenylalanine, phe; glutamic acid, glu.

Table 1 Isua rocks and lichen species amino acid D/L ratios

Sample	Extraction	Concentration†	Glu	Phe	Asp	Pro	Leu	Ala	Ile	Val
3016	H ₂ O	26.6	0.269	‡	0.177	0.192	0.115	0.220	0.053	0.073
3016	HCl	49.2	0.158	‡	0.127	0.066	0.023	0.088	‡	§
3022	H ₂ O	8.0	0.141	‡	0.162	0.181	0.061	0.108§	‡	‡
3022	HCl	13.4	0.076‡	‡	0.124	0.054	§	‡	‡	‡
3028	H ₂ O	62.2	0.186	0.064	0.150	0.127	0.094	0.206	0.028	0.068
3028	HCl	7.9	§	§	0.194§	0.045	0.140§	0.174	‡	§
155782*	H ₂ O	1.3	0.087‡	‡	0.178	0.229	‡	0.340	‡	‡
155782*	HCl	3.6	‡	‡	‡	‡	‡	‡	‡	‡
<i>Rhizocarpon</i> sp.	HCl	4.5	0.034	0.023	0.059	0.052	0.032	0.022	‡	0.027
<i>Dermatocarpon</i> sp.	HCl	104.0	0.033	0.024	0.050	0.035	0.027	0.029	‡	0.027
<i>Pseudophebe minuscula</i>										
Free fraction	HCl	0.1	0.025	‡	‡	0.035	‡	‡	‡	‡
Bound fraction	HCl	1.6	0.028	‡	0.048	0.023	0.029	0.026	‡	0.017

Approximately 2.0% of the D-amino acids used to obtain the D/L ratios may be attributed to (–)-2-butanol impurities and racemization during 6M HCl hydrolysis. Average instrumental error of three gas chromatographic determinations for each D/L ratio = ± 0.011 . Asp D/L ratios could not be corrected for minor γ -aba interference.

* This rock specimen is another portion of the sample on which the original Pb/Pb date of the Isua rocks was determined³⁰. This age has been confirmed by other methods, see ref. 31.

† These values are total concentrations of the amino acids detected in the samples. The Isua extracts = nmol g⁻¹; the lichen extracts = nmol mg⁻¹ $\times 10^{-2}$. The experimental error for the amino acid concentration is $\pm 20\%$.

‡ D peak and/or L peak too small to determine D/L ratios. The two values reported are based on only single gas chromatographic determinations.

§ Interference of D peak and/or L peak.

|| Glu and Phe were not included in this value because of gas-chromatographic peak interference.

The water-extracted rock residues were digested for 24 h at 100 °C in 6M HCl. The HCl solutions were analysed by the same procedure as above. During the HCl digestion the possibility arose that release of ferric iron or other substances may have oxidized the amino acids. To test this, an 8.1-g portion of sample 155782 was mixed with L-norvaline (1.7 mg) and L-norleucine (1.9 mg), two amino acids not originally detected in 155782. The mixture was digested and analysed as before. Approximately 75% of these amino acids were recovered after HCl digestion. No racemization was noted. 6M HCl extracts of two lichen species from the surface of sample 3016 (*Rhizocarpon* sp., *Dermatocarpon* sp.) and one lichen species from 3028 (*Pseudophebe minuscula*) were analysed for amino acids. Before acid hydrolysis *P. minuscula* was sonicated for 30 min with 1.5 M HCl to liberate non-bound amino acids and/or peptides. This free fraction was hydrolysed separately.

Figure 1 shows the results of the analysis of sample 3028 and of the 6M HCl extract of *P. minuscula*. Notable similarities exist between the relative abundances of amino acids in the lichens and in the Isua rocks. Glutamic acid, aspartic acid and glycine, the most abundant amino acids in the three lichen species analysed, were also the most abundant amino acids in the Isua rocks. Co-injection and GC-CIMS confirmed the presence of small quantities of four uncommon non-protein amino acids (α -amino-*n*-butyric acid, β -amino-*n*-butyric acid and/or β -aminoisobutyric acid, γ -amino-*n*-butyric acid and sarcosine) in the 3028 water extract. Three of these amino acids have been detected in biological systems⁶; β -amino-*n*-butyric acid and β -aminoisobutyric acid can also be derived biologically. α -Amino-*n*-butyric acid and γ -amino-*n*-butyric acid can evolve by the decomposition of threonine and glutamic acid, respectively⁷⁻⁹.

D/L ratios and the abundances of the amino acids from the Isua rocks and lichens were determined by gas chromatography and are listed in Table 1. Part of the Isua 3016 water extract that was analysed on an amino acid analyser revealed the presence of threonine, serine, tyrosine, histidine, arginine and lysine (P. E. Hare, personal communication), in addition to the amino acids listed for sample 3016 in Table 1. In temperate environments containing moisture, syngenetic amino acids should be substantially racemized (or epimerized) after ~a few hundred thousand to a few million years¹⁰⁻¹³. The amino acids found in the ~3,800-Myr-old Isua metamorphosed sedimentary rocks were not racemic. The high L-amino acid abundances introduced by modern lichens may have lowered the D/L ratios of older amino

acids in the Isua rocks. Amino acids extracted from the Isua rocks with 6M HCl seem to be more tightly bound to the organic and/or inorganic rock matrices than the water-extractable amino acids; they yielded lower D/L ratios. This is not surprising considering recent reports on the adsorption of amino acids by organic and inorganic rock constituents and their concomitant effects on amino acid racemization rates¹⁴⁻¹⁷.

After deposition, the Isua rocks experienced episodes of metamorphism. During the metamorphic events temperatures were 400–600 °C (refs 18, 19). Elevated temperature experiments have demonstrated that amino acids and peptides rapidly decompose when exposed to temperatures within the range of the Isua metamorphism^{20,21}. Serine, threonine and tyrosine are particularly unstable at elevated temperatures and in geological environments^{22,23}. Their presence further precludes an elevated temperature history for the amino acids in the Isua rocks.

To test the possibility of diffusion of amino acids from the lichens into the rocks, fragments of samples 3016 and 3015 were

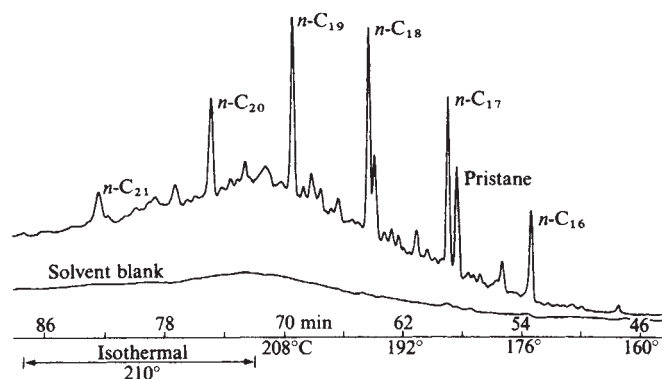


Fig. 2 Gas chromatograms of the procedure blank and the saturated hydrocarbons in sample 3016. The alkane distribution pattern may suggest some antiquity and/or contribution from a petroleum distillate fraction. For gas chromatography, an Apiezon L 15 m $\times$ 0.5 mm i.d., WCOT stainless-steel column was used. The GC programme was 80 °C isothermal for 6 min; then 80–210 °C at 2 °C min⁻¹. The He carrier gas flow rate was 3.5 ml min⁻¹. The inlet temperature was 195 °C. The chromatograms shown above were obtained using a Perkin Elmer F-11 gas chromatograph. In addition to co-injection of standards, the major hydrocarbon components in samples 3016 and 3028 were identified by GC-MS.

placed in aqueous solutions of D-amino acids for 1,321 h at 36 °C. After soaking, the concentrations of the D-amino acids in the rock interiors were 1 or 2 orders of magnitude higher than before exposure. Some amino acids present in the Isua rocks were probably introduced by encrusting lichens. However, the lichens chosen for analysis in this study were apparently not the direct source of the less common amino acids such as α -amino-*n*-butyric acid, β -amino-*n*-butyric acid/ β -aminoisobutyric acid and sarcosine which were present in low concentrations in the Isua rocks.

Comparisons of the Isua rock amino acid D/L ratios with amino acid D/L ratios reported for samples from other regions that have experienced comparable cold-temperature histories, such as the late Pleistocene fossil shells from the eastern Canadian Arctic²⁴ and shells from a Caribbean core (bottom water = 4 °C)²⁵, indicate that some of the Isua amino acids may be several tens of thousands of years in age; although comparisons of amino acid D/L ratios in rocks with fossil shells are tenuous. Comparisons of the amino acid D/L ratios of the Isua rocks with those reported from clay-rich (low-carbonate) sediments from Leg 15, Site 148, of the Deep Sea Drilling Project²⁶ and with amino acid D/L ratios reported for siliceous oozes from a core taken from the southern Atlantic Ocean (lat 56°34.7' S, long 20°17.2' W; bottom water, -0.2 °C)²⁷ further suggest that none of the amino acids in the Isua rocks are older than a few tens of thousands to possibly a few hundreds of thousands of years.

Interior portions of Isua samples 3016 and 3028 were also analysed for saturated hydrocarbons. The rock powders were first extracted with H₂O at 100 °C followed by CHCl₃/CH₃OH (87/13, v/v) at room temperature. Two alumina columns (Woelm Neutral, Waters Associates) were prewashed with distilled *n*-hexane. Procedure blanks and sample extracts were eluted from the alumina columns with *n*-hexane. The samples and blanks were analysed by gas chromatography and GC-MS.

Gas chromatograms of the procedure blank and hydrocarbon extract of sample 3016 are shown in Fig. 2. A similar hydrocarbon envelope was detected from sample 3028. The aliphatic hydrocarbons in samples 3016 and 3028 ranged from *n*-C₁₆ to *n*-C₂₁. Pristane was present in both samples. No odd carbon number preference was found for these Isua hydrocarbon extracts, indicating that these hydrocarbons were old or were introduced more recently from some organisms lacking odd carbon preference. Alternatively, they may have been derived from older materials brought into contact with the rocks by glacial action or by contamination with petroleum products.

It may clarify the origin of the hydrocarbons found in the Isua rocks¹ if pertinent laboratory experiments and related kinetic and thermodynamic calculations are considered. Elevated temperature experiments have shown that, at 375, 400 and 600 °C, the temperature range of the Isua metamorphic episodes, straight chain aliphatic hydrocarbons decompose in ~6½ days, ~5 days and in ~10 s, respectively^{28,29}. In experiments conducted in this laboratory, pristane (10 µl); pristane (7 µl) and graphite (10.2 mg); and pristane (6 µl) and a Santa Barbara Basin Recent marine sediment (3.7 mg) were heated in glass tubes under He at 520 °C for 2,924, 1,891 and 2,107 h, respectively. Next, the samples were extracted with *n*-hexane by ultrasonication and analysed by GC-MS. Pristane was destroyed in all three samples. ΔG for the decomposition of nonadecane (C₁₉H₄₀ → 19C + 20H₂) at 427 °C is -212.68 kcal mol⁻¹, (S. L. Miller, personal communication). ΔG for the decomposition of naphthalene (C₁₀H₈ → 10C + 4H₂) at 427 °C is -79.45 kcal mol⁻¹, and for biphenyl (C₁₂H₁₀ → 12C + 5H₂) at 427 °C is -101.63 kcal mol⁻¹. This indicates that even aromatic hydrocarbons, if initially present, may not have survived prolonged Isua metamorphic episodes. Thus the limited thermal stabilities of alkanes, these aromatic hydrocarbons, amino acids, and the low amino acid D/L ratios, probably preclude a very great age.

We thank S. Moorbath for a rock sample and for reviewing the manuscript, and M. Schidlowski for rock samples and helpful

comments, T. Nash for identifying the lichen species. P. E. Hare for help with the amino acid analyser analyses, E. S. Barghoorn for helpful comments and J. L. Bada and G. Eglinton for reviewing the manuscript. This research was supported by NASA grant NGR 03-002-171.

Received 21 July; accepted 8 October 1980.

1. Walters, C. et al. *Abstr. 178th Am. chem. Soc. A. Meet.*, Part 1, Washington, DC Geoc. 5 (1979).
2. *Chem. Engng News* 75, No. 38, 22-23 (1979).
3. Core Laboratories, *Rep.* 79072-1 (1979).
4. Engel, M. H., Zumberge, J. E. & Nagy, B. *Analyt. Biochem.* 82, 415-422 (1977).
5. Zumberge, J. E., Engel, M. H. & Nagy, B. in *Biogeochemistry of Amino Acids* (eds Hare, P. E., Hoering, T. C., King, K. Jr) 503-525 (Wiley, New York, 1980).
6. Meister, A. *Biochemistry of the Amino Acids* Vol. 1 (Academic, New York, 1965).
7. Schroeder, R. A. *Geol. Soc. Am. Abstr. Prog.* 9, 1163 (1977).
8. Bada, J. L. & Shou, M.-Y., *Geol. Soc. Am. Abstr. Prog.* 9, 885-886 (1977).
9. Povolo, D. & Vallentyne, J. R. *Geochim. cosmochim. Acta* 28, 731-734 (1964).
10. Abelson, P. H. & Hare, P. E. *Yb. Carnegie Instn Wash.* 67, 208-210 (1969).
11. Bada, J. L. *Earth planet. Sci. Lett.* 15, 223-231 (1972).
12. Kvenvolden, K. A. *Space Life Sci.* 4, 60-68 (1973).
13. Dungworth, G. *Chem. Geol.* 17, 135-153 (1976).
14. Carter, P. W. *Geochim. cosmochim. Acta* 42, 1239-1242 (1978).
15. Carter, P. W. & Mitterer, R. M. *Geochim. cosmochim. Acta* 42, 1231-1238 (1978).
16. Bondy, S. C. & Harrington, M. E. *Science* 203, 1243-1244 (1979).
17. Hare, P. E. & Hoering, T. C. *Yb. Carnegie Instn Wash.* 76, 625-631 (1977).
18. Nagy, B., Nagy, L. A., Zumberge, J. E., Sklarew, D. S. & Anderson, P. *Precamb. Res.* 5, 109-120 (1977).
19. Appel, P. W. U. *Precamb. Res.* 11, 73-87 (1980).
20. Vallentyne, J. R. *Geochim. cosmochim. Acta* 28, 157-188 (1964).
21. Simmonds, P. G., Shulman, G. P. & Stenbridge, C. H., *J. chromatogr. Sci.* 7, 36-41 (1969).
22. Hare, P. E. *Yb. Carnegie Instn Wash.* 75, 801-806 (1976).
23. Williams, K. M. & Smith, G. G. *Origins of Life* 8, 91-144 (1978).
24. Miller, G. H. & Hare, P. E. *Yb. Carnegie Instn Wash.* 74, 612-617 (1975).
25. Kvenvolden, K. A., Peterson, E., Wehmiller, J. & Hare, P. E. *Geochim. cosmochim. Acta* 37, 2215-2225 (1973).
26. Bada, J. L. & Man, E. H. *Earth Sci. Rev.* (in the Press).
27. Warnke, D. A., Blunt, D. J. & Pollock, G. E. in *Biogeochemistry of Amino Acids* (eds Hare, P. E., Hoering, T. C. & King, K., Jr) 183-189, (Wiley, New York, 1980).
28. Henderson, W., Eglinton, G., Simmonds, P. & Lovelock, J. E. *Nature* 219, 1012-1016 (1968).
29. Abelson, P. H. in *Researches in Geochemistry* (ed. Abelson, P. H.) 79-103 (Wiley, New York, 1959).
30. Moorbath, S., O'Nions, R. K. & Pankhurst, R. J. *Nature* 245, 138-139 (1973).
31. Hamilton, P. J., O'Nions, R. K., Evensen, N. M., Bridgwater, D. & Allaart, J. H. *Nature* 272, 41-43 (1978).

Preclassic lowland maize from Cuello, Belize

Charles H. Miksicek*, Robert McK. Bird†, Barbara Pickersgill‡, Sara Donaghey§, Juliette Cartwright¶ & Norman Hammond§

* Arizona State Museum and Department of Arid Lands Studies, University of Arizona, Tucson, Arizona 85721

† Institute for the Study of Plants, Food and Man, PO Box 9983, Kirkwood, Missouri 63122

‡ Department of Agricultural Botany, University of Reading, Reading RG6 2AS, UK

§ Archeological Research Program, Douglass College, Rutgers University, New Brunswick, New Jersey 08903

¶ Department of Anthropology, University of Texas, Austin, Texas 78712

In spite of recent progress towards an understanding of pre-hispanic Maya agriculture, relatively little is known about plants grown prehistorically in the Petén, Belize and the Yucatan Peninsula. Little plant material has been reported from Maya sites, and almost no detailed archaeobotanical analyses have been done. The Cuello site (18°05'N, 88°35'W) in northern Belize is unique in having numerous intact floors of very early ceremonial structures which separate plant remains into clearly defined units. Flotation of soil samples has yielded a wide range of carbonized plant material including many maize cupules (cob fragments) and kernels. As we report here, these can be classified into several types which seem to relate to a range of archaeological types of maize found elsewhere.

Table 1 Preclassic Cuello maize data

No.	Row no.	Cupule width	Cupule length	Cupule wing width	Rachis diameter [†]	Cupule thickness	Cupule depth	% Of all maize from each phase			
								Swasey	Mamom	Chicanel	
<i>Swasey type 1 (2000 b.c.–a.d. 250)</i>											
Mean 12	12.8	2.4	1.6	0.8	5.2	1.7	1.2	31	6	2	
σ	3.6	0.24	0.33	0.20		0.68	0.29				
<i>Swasey type 2 (2000 b.c.–a.d. 250)</i>											
Mean 49	12.9	2.9	1.7	0.9	6.2	2.0	1.1	42	35	28	
σ	2.4	0.23	0.24	0.23		0.36	0.48				
<i>Swasey type 3 (2000 b.c.–a.d. 250)</i>											
Mean 44	11.5	3.6	2.1	1.2	7.2	2.3	1.2	27	41	27	
σ	2.1	0.27	0.32	0.37		0.55	0.44				
<i>Mamom type 1 (1000 b.c.–a.d. 250)</i>											
Mean 13	11.4	4.6	2.2	1.3	8.6	2.9	1.3		14	10	
σ	2.6	0.15	0.27	0.21		0.21	0.21				
<i>Mamom type 2 (1000 b.c.–a.d. 250)</i>											
Mean 7	10.0	5.9	2.5	1.9	10.1	3.0	1.4		4	8	
σ	1.3	0.49	0.50	0.29		0.57	0.40				
<i>Chicanel type 1 (400 b.c.–a.d. 250)</i>											
Mean 16	12.3	4.6	3.0	1.8	9.3	3.5	1.2			25	
σ	2.7	0.38	0.40	0.29		0.66	0.31				

All measurements except row no. are in mm, not corrected for carbonization. Means and standard deviations of five measurements and two estimates for six Preclassic Cuello cupule categories. All the measurements (mm) are from carbonized specimens, so for comparison to uncarbonized specimens, about 25% needs to be added to the values.

* Row no. estimated: $360^\circ/\text{cupule angle}$, rounded off, multiplied by 2.

† Rachis diameter estimated by law of cosines: rachis diameter = cupule width $\times 1.414 (1 - \cos \text{cupule angle})^{-0.5}$.

Excavations at Cuello during 1976–1980 revealed superimposed early structures, some of probably ceremonial function^{1–3}. The overall chronology of the site is from about 2000 b.c. in radiocarbon years to about AD700 in calendar years, the early part of the Late Classic period of historically dated Maya civilization^{4,5}. (The Preclassic or Formative period before AD 250 is dated by a suite of 20 radiocarbon dates from the Cambridge (Q) and Los Angeles (UCLA) laboratories provided by V. R. Switsur and R. Berger; further samples are being processed by these two laboratories and also that at La Jolla (LJ) by H. Suess. (Lower case b.c. and a.d. indicate radiocarbon years with no correction for the bristlecone pine chronology, geomagnetic curve, or other factors.) The occupation of Cuello is formally divided into five successive phases, three in the Formative and two in the Classic period, based on the stratigraphy, pottery and radiocarbon dates from excavations principally in Platform 34 at the site. The Early Formative Swasey phase (2000–1000 b.c.) is succeeded by the Middle Formative Lopex Mamom phase (1000–400 b.c.) and the Late Formative Cocos Chicanel phase (400 b.c.–AD 250), then by the Early Classic Nuevo Tzakol (AD 250–600) and Late Classic Santana Tepeu (AD 600–700). Classic period deposits consist of superficial architecture and do not feature in the sampling programme reported here.

For the entire Formative period a succession of plaster floors define and separate layers of occupational material and fill. The possibility of vertical displacement of plant remains after deposition is minimal, although some animal and insect burrows were noted in the excavation and some root penetration was also present. The recycling of earlier material in later construction fills is, on the other hand, a reality attested to by abraded sherds of Swasey pottery occurring in Mamom and Chicanel contexts.

From 1978 to 1980 we carried out an intensive programme of sampling for plant remains by water flotation⁶, processing 250 50-l samples representing more than 10 metric tons of earth. Soil samples were sealed into plastic bags and before flotation were partially air-dried to enhance recovery of carbonized plant parts, mollusc shells and bones. The smallest materials were land snails and seeds whose longest dimensions are 0.5 mm, so that little recoverable and identifiable material was missed. We recovered *Zea mays* kernel, cupule and glume fragments from

all contexts including the earliest occupation on the old land surface dated at 1950 ± 65 b.c. (Q-1571)¹. From the samples analysed so far, 141 cupules (of a total of 329) and 56 kernels (of 821) were complete enough to measure in several ways. The lack of intact maize cobs may be due to breakage during construction of the platforms, predepositional handling including burning, and a low level of cob induration.

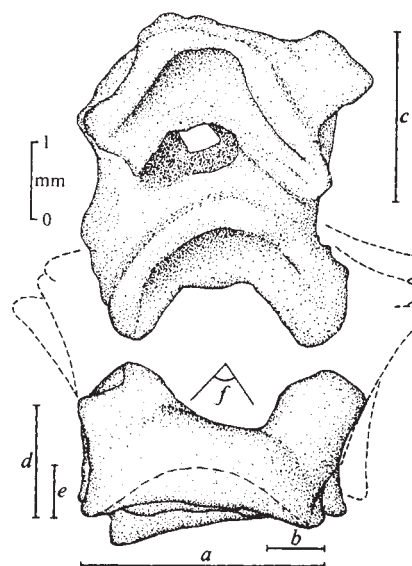


Fig. 1 A cob fragment with two almost complete cupules and one partial cupule to each side, similar to some Swasey type 2 cupules, dating to about 1000 b.c. Upper, side view; lower, end view (apical) demonstrating that the whole cob had five ranks of cupules, each subtending two fertile spikelets, giving ten rows of kernels. An estimate of the rachis outline and the interior of one cupule are indicated by dashes on the lower part of the figure. The measurements indicated are *a*, cupule width; *b*, cupule wing width; *c*, cupule thickness; *d*, cupule length; *e*, cupule depth, and *f*, cupule angle (used for estimating row number). (Drawn by R.McK.B.)

The variables we used to measure the cupules (Fig. 1) have been applied to a wide range of prehistoric and modern cobs⁷⁻¹¹. Cupule angle has been used by Cutler to estimate row number¹². Usually several measurements need to be used simultaneously to distinguish types of maize because races overlap in many metric values, often being differentiated by only one of the available variables. Several characters that are not easily quantifiable, such as cupule shape, may also aid in separation. Cuello maize types are named according to the phases in which they first appear.

The classification of extant races of maize is based on a broad spectrum of variables: morphology of the plant, tassel and ear; physiology; cytology; and most recently, isozymes of the coleoptile¹³⁻¹⁶. Because the Cuello collection consists of only charred kernel and cob fragments, much of the necessary evidence for complete racial classification is lacking. Nevertheless, it is important to attempt to understand the relationships of the Cuello maize to existing modern races and other archaeological collections.

The smallest type of Cuello maize is Swasey type 1 (SW-1). It has small elliptical to rectangular cupules with narrow cupule wings (Table 1), and 10-16 rows of grains, as estimated by cupule angle. The smallest kernels from Cuello, probably associated with this type, all have hard endosperm and probably represent a popcorn. They measure 4.2 × 3.2 mm (kernel width × kernel thickness, charred). Allowing for 20% shrinkage on carbonization, and estimating rachis diameter using the law of cosines (equation, Table 1), we believe that the intact ear would have been about 22 mm in diameter, about the size of a modern US popcorn called Ladyfinger. Without an entire cob, it is impossible to estimate length. SW-1 is more like early types of maize from South America (Cupisnique types 3 and 4, Gallinazo types 1 and 3) than any reported Mexican archaeological collections (Table 2).

Swasey type 2 (SW-2) and Swasey type 3 (SW-3) are somewhat similar, but can be distinguished by cupule width and shape. In outline SW-2 cupules are trapezoidal, chevron-shaped (Fig. 1) or B-shaped. SW-3 cupules tend to be more rectangular

with wide wings, some of which resemble small 'ears'. The cupules of SW-2 often have slight apical protrusions resembling the toe of a boot. This configuration (sometimes called 'shoe-shaped' or 'Spanish stirrup') is present in some Tehuacán specimens, as well as modern Chapalote and many South American races. SW-2 has 10-16 rows of kernels averaging 5.6 × 4.2 mm (charred) which resemble a rounded popcorn. SW-3 has a slightly lower mean row number (with a range of 8-16) and kernels measuring 6.2 × 4.8 mm (charred) which seem to represent both popcorn and hard flint types. SW-2 is somewhat like Abejas maize but is very like Gallinazo types 1 and 4 from the north coast of Peru and Guadalupe-Largas maize from Oaxaca. SW-3 also resembles Guadalupe-Largas but is more like Early Nal Tel B and Pre-Chapalote (Table 2).

Mamom type 1 (MA-1) is larger than Swasey maize groups. It has wide, but comparatively short, rectangular to 'eared' cupules with broad wings. Charred kernels apparently associated with this type are all flinty (probably a soft flint), measuring 6.4 × 4.6 mm (charred). Uncarbonized MA-1 cobs would have been about 16 mm in diameter and, with kernels, the ears may have been about 28 mm in diameter. MA-1 is almost identical to Early Nal Tel B from La Perra Cave (Table 2) and is similar to Early Teosintoid, Late Nal Tel and Early Chapalote. With the appearance of MA-1, the resemblance between the Cuello material and the listed Peruvian maize types diminishes. MA-1 was probably very similar to a previously undescribed Central American race which we have collected from Santa Cruz, August Pine Ridge and Rancho Creek (Orange Walk District) and Aguacate (Toledo District) in Belize. This race has 10-14 rows of soft flint or semi-hard dent kernels with white, purple, yellow or pale red aleurone. The ears average 32 mm in diameter and 106 mm long. Although local Mayan farmers refer to this race as 'Dzit Bacal', it bears no resemblance to published descriptions of Dzit Bacal from Guatemala or the Yucatan^{15,16}.

Mamom type 2 (MA-2) is the largest of all Preclassic Cuello maize varieties. MA-2 has deep, wide trapezoidal or chevron-shaped cupules with 8-12 rows of flinty kernels measuring 6.8 × 4.3 mm (charred). It resembles Early Nal Tel B and Late

Table 2 Formative period maize from Latin America

Area	Type	Time range	Mean kernel row no.	Mean cupule width (mm)	Mean cupule length (mm)	Mean cupule wing width (mm)	Mean rachis diameter (mm)	Mean cob length (mm)	Index of similarity to Cuello maize types					
									SW-1	SW-2	SW-3	MA-1	MA-2	CH-1
Tehuacán Valley	Coxcatlán	5000-3500 b.c.	7.6	3.0	2.7	0.4	3.5	23.5	17	33	17	17	17	0
	Abejas	3500-2300 b.c.	9.0	4.2	2.5	0.4	5.1	27.7	0	50	33	17	33	0
	Early Teosintoid	1500-900 b.c.	12.0	6.2	3.5	1.3	12.4	78.4	33	33	33	83	83	83
Sierra de Tamaulipas	Early Nal Tel A	2495-250 b.c.	8.9	5.4			8.4	48.0	0	33	33	33	33	33
	Early Nal Tel B	2495-250 b.c.	10.2	6.2	3.1	1.7	10.6	60.0	20	20	80	100	80	80
	Late Nal Tel	250 b.c.-a.d. 150	10.9	7.1	2.6	1.6	13.2	86.0	20	20	60	80	80	40
Infiernillo Canyon	Pre-Chapalote	2350-400 b.c.	9.0	4.8*	3.0	1.4	7.5	30.0	20	20	80	60	40	40
	Early Chapalote	1850 b.c.-a.d. 800	10.5	5.8*	4.1	1.8	9.9	58.4	20	20	60	80	20	100
Oaxaca Valley	Guadalupe-Largas	1200-550 b.c.	11.5	(3.3)	(3.2)		(6.6)*	25	75	75	25	0	0	50
Chicama Valley ⁸	Cupisnique-3	850-450 b.c.	15.3	2.6	1.7	0.5	7.3	49.5	80	40	20	0	0	0
	Cupisnique-4	850-450 b.c.	12.0	2.8	1.6	0.5	6.0	46.0	80	20	20	20	20	20
	Gallinazo-1	a.d. 20-220	15.1	2.9	1.8	0.5	7.6	45.0	80	80	20	0	0	0
	Gallinazo-2	a.d. 20-220	13.7	4.0	1.7	0.6	8.6	46.0	40	60	60	40	0	20
	Gallinazo-3	a.d. 20-220	10.2	2.9	1.7	0.5	5.3	36.0	80	20	20	20	20	0
	Gallinazo-4	a.d. 20-220	10.0	4.0	2.1	0.8	6.7	42.0	60	80	60	20	20	0
	Gallinazo-5	a.d. 20-220	11.1	5.4	1.7	1.0	9.5	62.0	60	60	40	60	20	40

Measurement means for early maize types from eight archaeological sites in five areas of Latin America. The data from Tamaulipas are taken from photographs and published information. The Tehuacán Valley (Puebla, Mexico) sites are the Coxcatlán and San Marcos Caves⁸; the Sierra de Tamaulipas (Mexico) site is La Perra Cave¹⁷; the Infiernillo Canyon (Mexico) sites are Romero's Cave, Valenzuela's Cave and Ojo de Agua¹⁸; the Oaxaca Valley (Mexico) site is Fabrica San José¹⁹; the Chicama Valley (Peruvian north coast) site is Huaca Prieta, including an associated area 150 m north⁸. The maize comes from two or more cultural phases at each site. An asterisk indicates that the value for rachis diameter is estimated as explained in Table 1, or that cupule width is estimated from rachis diameter. Figures in parentheses are from carbonized specimens with about 20% shrinkage. Cupule depth for the five Coxcatlán cobs is 0.7 mm, and for the four Abejas cobs is 1.1 mm. Cupule thickness for Coxcatlán maize averages 1.7 mm. The index of similarity is calculated by adding 25% to the values for carbonized Cuello specimens, then establishing a ±20% range around these adjusted values, counting overlaps of this range with the mean values per type, and dividing by the number of possible comparisons.

Nal Tel from La Perra Cava and Early Teosintoid maize from Tehuacán (Table 2). Before carbonization, the cobs of MA-2 would have been about 20 mm in diameter and with kernels the ears would have measured about 30 mm in diameter. It was probably quite similar to the Tuxpeño-introgressed Nal Tel grown by traditional *milperos* throughout Belize today.

Approximately three-quarters of the Cuello Chicanel-1 material comes from Square 45/35 Layer 1597, a burnt house (apparently with a corn crib) dating to the early Chicanel phase, about 250 b.c. Because all the maize from this context was quite similar, it probably represents a single type from one harvest. CH-1 has open, trapezoidal, 'Spanish-stirrup' cupules with broad cupule wings and 8–16 rows of flinty kernels measuring 6.5 × 5.0 mm (charred). It is almost identical to Early Chapalote from Tamaulipas and is quite similar to Early Teosintoid and Early Nal Tel B (Table 2).

Because they lack all but cob and kernel fragments, we know nothing about ear length, plant form, productivity or other characters of these early lowland maize types. Although the general increase in rachis diameter and kernel size through time suggests an increase in productivity, we must be cautious in making this assumption. A single plant with many small ears (which is true of many popcorns and primitive maize types) could produce as much food as a plant with one or two larger ears. Perhaps evidence for other plant characters and the early adaptation of maize to the lowlands will be found in dry caves in arid valleys along the upper Motagua drainage in southeastern Guatemala.

The earliest domesticated maize was probably adapted to the semi-arid highlands of central and southern Mexico. By at least 2000 b.c. a distinct lineage including SW-1 and SW-2 was developed which was more adapted to the humid lowlands. This lineage probably spread throughout tropical Central America and into coastal South America and contributed to the development of sedentary horticulturalists such as the Swasey people at Cuello. After 1000 b.c. the relationship of Cuello maize to highland Mexican types is more pronounced. The improvement of earlier Swasey maize and hybridization with highland germplasm (and possibly teosinte) produced new varieties which eventually helped to support a dense population and the complex culture of the Maya lowlands.

Major funding for this field work came from the National Geographic Society, the British Museum, the Society of Antiquaries of London, the Wenner-Gren Foundation for Anthropological Research Inc. and Rutgers University. Valuable field assistance was given by Ingrid Wuebbler, Kathryn Elssesser, and other members of the Cuello Project Staff; John Masson and Richard Wilk assisted with ethnobotanical collection of modern Maya maize. We thank the Director of the Museo Regional de Puebla and Mario Camacho Robles, Curador General, for access to collections of archaeological maize from the Tehuacán Valley, Puebla, Mexico.

Received 4 February; accepted 31 October 1980.

- Hammond, N. (ed.) *Cuello Project 1978 Interim Report*. Publ. no. 1 (Archaeological Research Program, Douglass College, Rutgers University, 1979).
- Hammond, N. *Belizean Stud.* 8, 33–44 (1980); *Belizean Stud.* (in the press).
- Hammond, N. et al. *Am. Antiq.* 44, 92–110 (1979).
- Hammond, N. et al. *Nature* 260, 579–581 (1976); 267, 608–610 (1977).
- Hammond, N. *Am. Antiq.* 44, 92–110 (1979).
- Minnis, P. E. & LeBlanc, S. *Am. Antiq.* 41, 491–493 (1976).
- Bird, R. McK. thesis, Univ. California, microfilm 71–9767 (1970).
- Bird, R. McK. *Maize Genet. Coop. News Lett.* 52, 90–92 (1978).
- Bird, R. McK. *Maize Genet. Coop. News Lett.* 53, 53–54 (1979).
- Bird, R. McK. & Bird, J. B. *Am. Antiq.* 45, 325–332 (1980).
- Nickerson, N. H. *Ann. Mo. Bot. Garden* 40, 79–111 (1953).
- Cutler, H. C. *Univ. Utah Anthr. Pap.* 80, 10–11 (1966).
- Bird, R. McK. & Goodman, M. M. *Econ. Bot.* 31, 471–481 (1978).
- Smith, J. S. C. & Lester, R. N. *Econ. Bot.* 34, 201–218 (1980).
- Wellhausen, E. J. et al. *Races of Maize in Mexico* (Bussey Institute, Harvard University, Cambridge, 1952).
- Wellhausen, E. J. *Races of Maize in Central America*, Publ. 511 (National Academy of Sciences-National Research Council, Washington DC, 1957).
- Mangelsdorf, P. C. et al. *Bot. Mus. Leaflet*, Harvard Univ. 17, 125–150 (1956).
- Mangelsdorf, P. C. et al. *Bot. Mus. Leaflet*, Harvard Univ. 22, 33–62 (1967).
- Ford, R. I. in *Prehistory and Human Ecology of the Valley of Oaxaca* (ed. Flannery, K. V.) Mem. Mus. Anthr. Univ. Mich. no. 8, 4, 261–268 (1976).

Electrical activity following cellular recognition of self and non-self in a sea anemone

Roger Lubbock

Department of Zoology, Downing Street, Cambridge CB2 3EJ, UK

G. A. B. Shelton

Department of Zoology, South Parks Road, Oxford OX1 3PS, UK

The sea anemone *Anthopleura elegantissima* lives in clonal colonies^{1–3} and possesses a cellular recognition system of remarkable specificity⁴, by which it can recognize members of its own clone; other anemones, including individuals of the same species which are not syngeneic, are attacked. Attack is initiated by contact with a foreign anthozoan and involves the inflation of specialized tentacle-like structures known as acrorhagi, which contain numerous stinging cells^{1,2}. These stinging cells only discharge when the tip of the acrorhagus is in physical contact with the surface of a foreign anthozoan; contact with syngeneic individuals, organisms other than anthozoans and inanimate objects does not elicit discharge⁴. We show here that the recognition of allogeneic tissue is accompanied by a novel form of local electrical activity in the acrorhagus that is usually, but not invariably, followed by nematocyst discharge. This type of electrical activity was not found during contact with syngeneic tissue or inanimate objects and seemed to be a consequence of the recognition of allogeneic surface markers by cells at the tip of the acrorhagus.

Clones of *A. elegantissima* were maintained in several 150–400 l aquaria containing artificial sea water (Tropic Marin Neu; relative density 1.025) at 23 °C. Before experimentation, an anemone was placed in a small bowl of sea water and induced to inflate its acrorhagi by contact with an allogeneic individual. Extracellular suction electrodes^{5,6} (tip diameter 200 µm) were applied to a point about half way along the inflated acrorhagus; signals were amplified by Tektronix Type FM 122 differential preamplifiers and displayed on a Tektronix Type 5111 storage oscilloscope and a Washington curvilinear pen recorder. Care was taken to maintain the electrodes in a single position when comparing responses. Discharge of nematocysts was observed using a binocular microscope, usually with dark ground illumination.

Touching the acrorhagus with the intact surface of excised syngeneic tentacles consistently failed to induce either nematocyst discharge or any recordable electrical excitation; in addition, no detectable activity could be produced by touching the acrorhagus with inanimate objects (metal forceps or glass rod). On the other hand, contact of the acrorhagial tip with an allogeneic tentacle required only a short delay before it produced massive, simultaneous nematocyst discharge, including numerous nematocysts remote from the point of contact (Fig. 1a, b); discharge was accompanied by a triphasic spike up to 50 µV in amplitude and about 350 ms in duration (Fig. 2). The precise timing of this spike in relation to that of nematocyst discharge was not established and it was therefore unclear whether the spike initiated discharge or was a consequence of it. Of greater interest in the present context was the occurrence of a variable number of much smaller impulses of up to 10 µV and up to 50 ms duration, which preceded the spike. It was particularly notable that these small impulses could also be evoked by brief contact with allogeneic material, even though no discharge of acrorhagial nematocysts and no spike occurred (Fig. 2). These impulses could not be ascribed to any of the known sea anemone electrical conduction systems⁷; they were distinctive in that they seemed to be confined to a single acrorhagus and could be

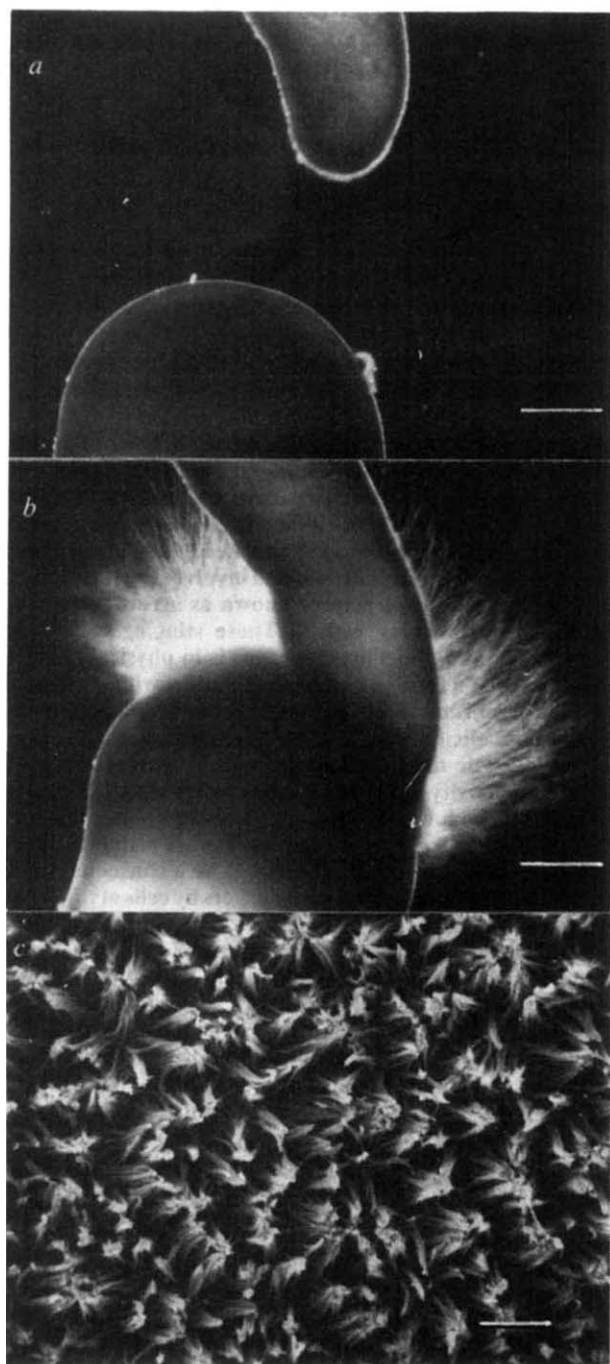


Fig. 1 *a*, Acrorhagus (below) and allogeneic tentacle (above) before contact. Scale bar, 300 μ m. *b*, Massive, simultaneous discharge of acrorhagial nematocysts after contact between acrorhagus (below) and allogeneic tentacle (above); discharge usually occurred within 0.5–3 s of contact. Scale bar, 300 μ m. *c*, Scanning electron micrograph of ciliary cones on the tip of an inflated acrorhagus. Scale bar, 5 μ m.

shown, using two electrodes, to decrease rapidly in amplitude at increasing distance from the acrorhagial tip. They were clearly not the result of the foreign tentacle stinging the acrorhagus: the impulses could also be obtained using fresh pieces of allogeneic column tissue (largely devoid of nematocysts), as well as washed foreign tentacles previously fixed in formalin. It thus seemed that the small impulses were a consequence of cells at the acrorhagial tip recognizing allogeneic markers on the foreign tissue.

The receptors associated with recognition were presumed to reside on the ciliary cones that densely cover the distal portion of the acrorhagus (Fig. 1c); it was notable that touching the

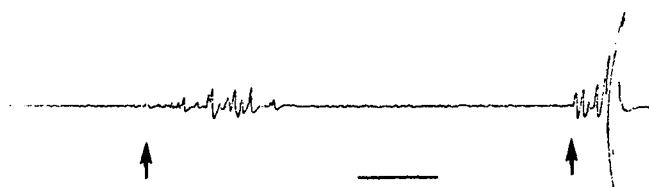


Fig. 2 Pen recording showing electrical response of acrorhagus to contact with an allogeneic tentacle. The beginning of each period of contact is indicated by an arrow; duration of contact was ~1 s. The first period of contact (left-hand arrow) evoked small impulses but no nematocyst discharge. The second period of contact (right-hand arrow) evoked small impulses which were succeeded by a large triphasic spike; the spike was accompanied by massive nematocyst discharge. Scale bar, 1 s.

unciliated sides and base of the acrorhagus with allogeneic tissue failed to produce any detectable electrical activity.

We suggest the following hypothesis of the means by which recognition of a foreign anemone leads to nematocyst discharge: specific molecules on the surface of the allogeneic tissue bind to surface receptors on cells at the tip of the acrorhagus, inducing electrical excitation; local events of this kind summate until a threshold is reached, at which point massive nematocyst discharge occurs. The fact that electrical activity was recorded on contact with allogeneic tissue, but not with syngeneic tissue of inanimate objects, is consistent with the previously advanced theory⁸ that self recognition in anemones is an essentially passive process, with specific receptors failing to be excited, whereas non-self recognition is an active process involving receptor excitation.

We thank W. B. Amos and G. Koch for commenting on the manuscript.

Received 18 July; accepted 24 October 1980.

1. Francis, L. *Biol. Bull.* **144**, 64–72 (1973).
2. Francis, L. *Biol. Bull.* **144**, 73–92 (1973).
3. Francis, L. *Am. Zool.* **19**, 669–681 (1979).
4. Lubbock, R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6667–6669 (1980).
5. Josephson, R. K. *J. exp. Biol.* **45**, 305–320 (1966).
6. Macfarlane, I. D. *J. exp. Biol.* **51**, 377–385 (1969).
7. Shelton, G. A. B. in *Electrical Conduction and Behaviour in "Simple" Invertebrates* (ed. Shelton, G. A. B.) (Oxford University Press, 1980).
8. Lubbock, R. *J. exp. Biol.* **83**, 283–292 (1979).

Surface antigen differentiation during human myogenesis in culture

Frank S. Walsh

Muscular Dystrophy Research Laboratories, Institute of Neurology, Queen Square, London WC1N 3BG, UK

Mary A. Ritter

Membrane Immunology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK

The fusion of mononucleate myoblast cells into multinucleate myotubes (myogenesis) has often been studied as a model system of terminal cellular differentiation¹. Although cell-surface changes during myogenesis have attracted much attention and a variety of surface antigens including the nicotinic cholinergic receptor², acetylcholinesterase³ and muscle lectins^{4,5} have been shown to be present on muscle cells, a detailed identification of markers specific to the various cell types has not been attempted. This is mainly because fibroblasts are a major contaminating cell type in primary muscle cultures and these cells have no known distinctive cell-surface antigen(s). For instance, surface fibronectin has been used to distinguish fibroblasts in the rat peripheral nervous system *in vitro*⁶ but has proved ineffective in the human muscle cell system⁷. In addition, Lesley and Lennon⁸ found that Thy-1 antigen was present on myoblasts but not myotubes of the rat L6 muscle cell line and primary cultures. However, Thy-1 antigen is also present on

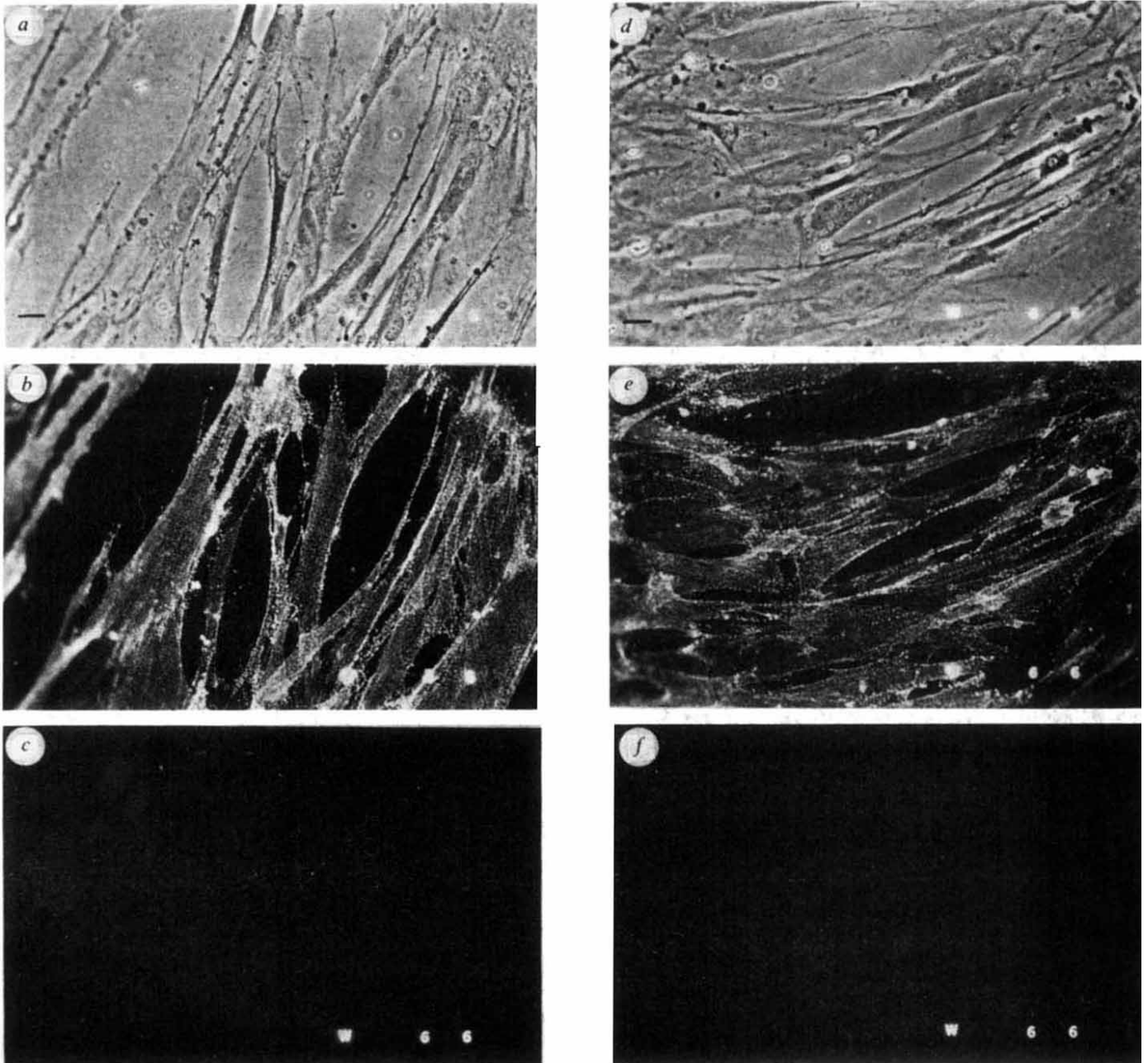
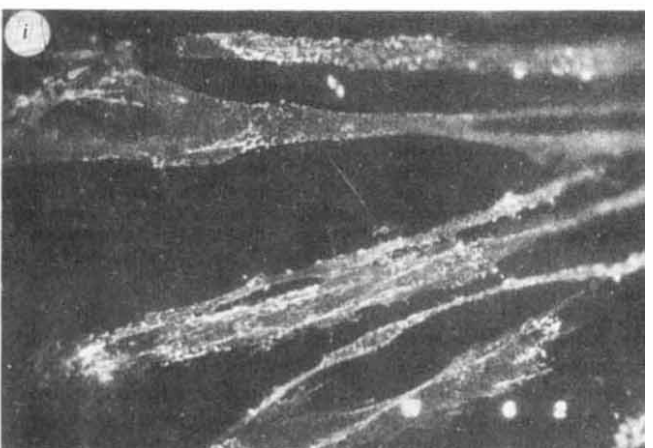
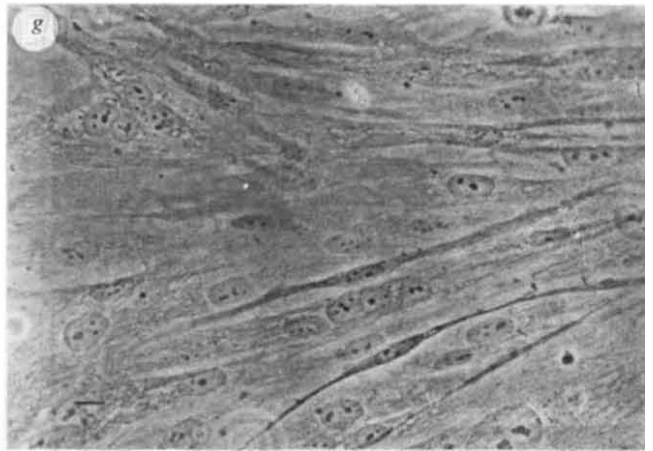
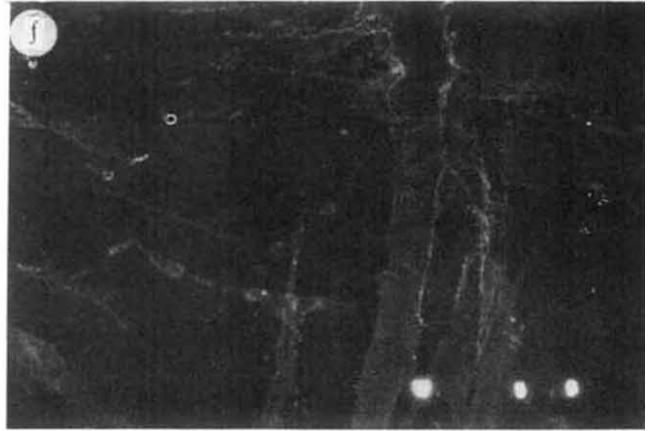
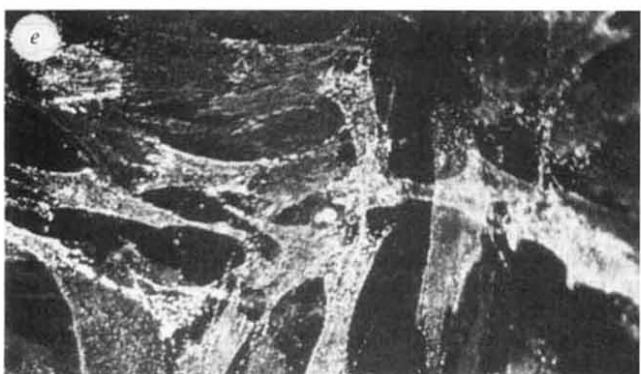
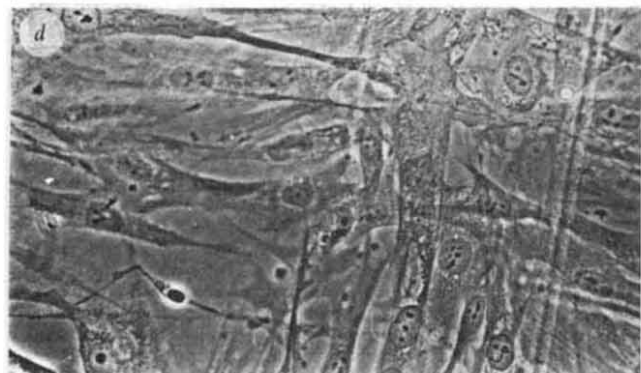
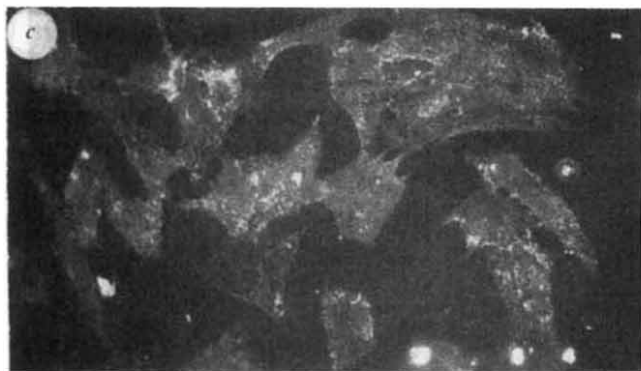
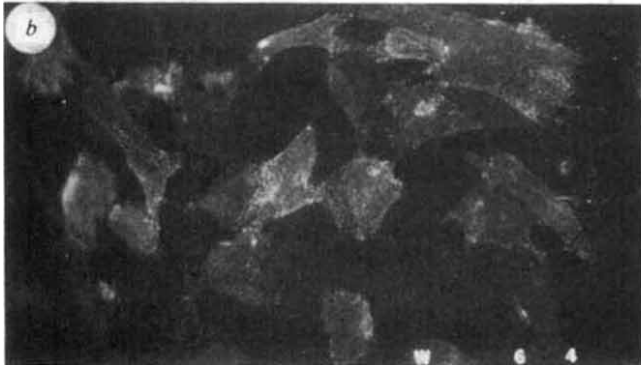


Fig. 1 Double immunofluorescent staining of fetal human skin fibroblast or muscle fibroblast cells with rabbit anti-rat Thy-1 antibody and 5.1 H11 monoclonal antibody. Fibroblasts were prepared from fetal skin by dissociating with trypsin and collagenase, using the procedure previously described for muscle¹⁷. The cells were grown on 60-mm Petriperm plates (Heraeus) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Fetal human muscle fibroblasts were prepared by preplating¹⁴ a single-cell suspension of enzymatically dissociated skeletal muscle. The cells that adhered to uncoated 100-mm Lux Petri dishes after 30 min incubation were regarded as muscle fibroblasts. These cells were grown to confluence in DMEM, 10% FCS, at least twice before use to allow any residual myoblasts to fuse into myotubes. These cultures express negligible activity of the muscle enzymatic marker creatine phosphokinase when grown to confluence. Immunofluorescent staining of skin or muscle fibroblast cultures was carried out on subconfluent cultures. The Petriperm plates were cut into 10-mm² pieces and 10 μ l of an F(ab')₂ preparation of rabbit anti-rat Thy-1 antibody (24 μ g ml⁻¹) in phosphate-buffered saline (PBS, 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.3) and 10 μ l of 1:100 dilution of 5.1 H11 antibody (ascitic fluid) in PBS added for 45 min at 20 °C. The cells were washed in PBS and 10 μ l of an F(ab')₂ fragment of sheep anti-rabbit IgG antibody (60 μ g ml⁻¹) labelled with rhodamine plus 10 μ l of a 1:10 dilution of goat anti-mouse IgG antibody labelled with fluorescein (Meloy) added for 45 min at 20 °C. The cells were washed extensively in PBS, fixed in 3.5% formaldehyde in PBS for 5 min followed by 5% acetic acid in ethanol for 5 min at 20 °C. The samples were mounted on glass slides in glycerol and sealed with nail varnish. Samples were examined with a Leitz dialux 20 microscope equipped with phase contrast fluorescein and rhodamine optics and epi-illumination. *a*, Phase-contrast view of skin fibroblast culture; *b*, positive Thy-1 staining viewed with rhodamine optics; *c*, negative 5.1 H11 staining with fluorescein optics; *d*, phase-contrast view of muscle fibroblast culture; *e*, positive Thy-1 staining viewed with rhodamine optics; *f*, negative 5.1 H11 staining with fluorescein optics. Scale bar, 20 μ m.

rat fibroblasts^{8,9}. Thus, unequivocal identification of the major mononucleate cell types in muscle cultures, fibroblasts and myoblasts has not been possible. We report here the use of two surface antigens, Thy-1 and a muscle-specific antigen recognized by a monoclonal antibody, to identify unambiguously the four major types of cells present in human muscle cultures and to propose a model of cell-surface differentiation during human myogenesis.

Two antibodies were used in this study. The first is a rabbit antibody to purified rat brain Thy-1 (refs 10, 11) which we have used to detect the human homologue of Thy-1 (refs 12, 13). Preabsorption of this antibody with pure rat brain Thy-1 (1:3 molar ratio) abolished all immunofluorescence on human muscle cells (see below), showing the staining to be specific for the Thy-1 molecule. In addition, because previous absorption of the anti-Thy-1 with Thy-1.2 mouse thymocytes also abrogated



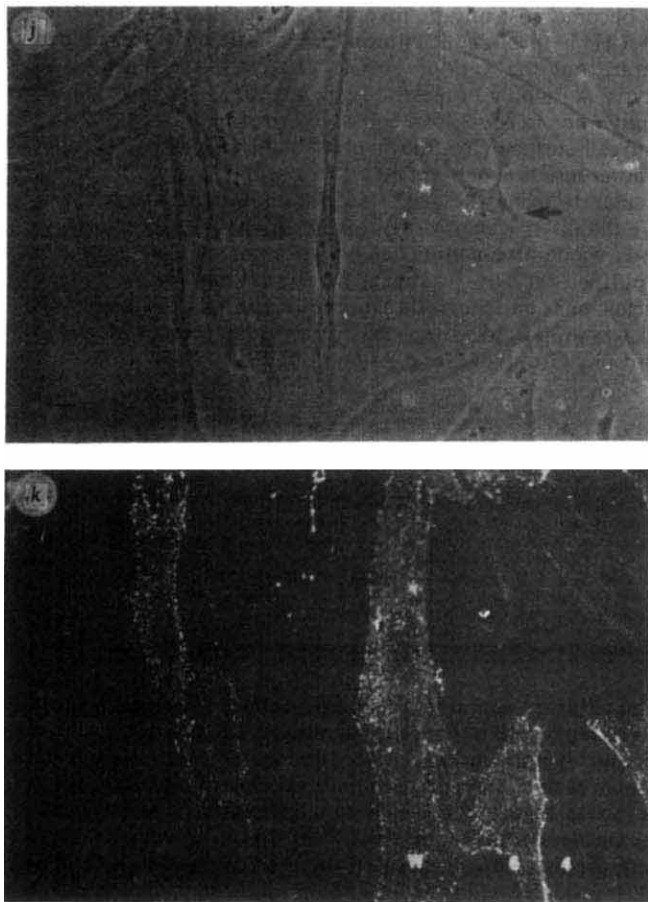


Fig. 2 Double immunofluorescent staining of fetal human muscle cultures at different time points during myogenesis with rabbit anti-rat Thy-1 antibody and 5.1 H11 antibody. Mononucleate muscle cells were prepared from fetal human tissue as described previously¹⁷. The cells were plated on to 60-mm Petriperm plates in the presence of DMEM, 10% FCS and 2% chick embryo extract (CEE). A myoblast time point (mononucleate cells) was taken 2-3 days after plating. *a* Shows a phase-contrast picture of the myoblasts and *b* and *c* the staining profile with Thy-1 and 5.1 H11 antibodies, respectively. Conditions for staining were exactly as in Fig. 1 legend. As the cultures grow and become dense, the cells begin to line up in a group and fuse into multinucleate myotubes. At this point the medium was changed to DMEM, 10% horse serum and 2% CEE to induce differentiation. A mid-fusion time point was taken 1 day after the medium change. At this stage myotubes were present but a large number of mononucleate cells remains. *d* Shows a phase-contrast photograph of a muscle culture at mid-fusion and *e* and *f* the same culture stained with anti-Thy-1 and 5.1 H11 antibody, respectively. As fusion progresses and the myotubes mature, a final post-fusion time point was taken (7-8 days after plating). At this stage most of the culture is taken up by large multinucleate myotubes but the muscle fibroblasts continue to divide and are also present. *g* Shows a phase-contrast photograph of a post-fusion muscle culture and *h* and *i* the same field stained with anti-Thy-1 and 5.1 H11 antibody, respectively. *j* Shows a phase-contrast photograph of a mid-fusion culture, and *k* and *l* the same field stained with anti-Thy-1 and 5.1 H11 antibodies, respectively. Note the mononucleate cell (see arrow) stained by 5.1 H11 antibody but not anti-Thy-1. Scale bar, 20 μ m.

all immunofluorescent staining on human cells, it must be the rat-mouse cross-reacting antigenic determinant that is shared by rat and human Thy-1. The second reagent, 5.1 H11 monoclonal antibody⁷, was produced from a cell fusion involving P3X63 Ag8 mouse myeloma cells and spleen cells from a BALB/c mouse immunized with human myotube cells. It reacts with human myoblasts and myotubes but not with fibroblasts and is human muscle cell specific⁷.

Human fetal skin fibroblasts, muscle fibroblasts and muscle cultures at different stages of myogenesis were grown and tested for reactivity with these antibodies by indirect immunofluorescence. Figure 1 shows that Thy-1 antigen is present on all cells in human skin and muscle fibroblast cultures; neither of these cell types showed detectable reactivity with 5.1 H11 antibody. The results with anti-Thy-1 antibody agree with data from rats that show Thy-1 antigen is expressed on fibroblasts⁹.

The distribution of Thy-1 and 5.1 H11 antigens in cultures of mononucleate muscle cells is shown in Fig. 2*a-c*. In contrast to the results with skin or muscle fibroblasts, the majority of the cells in the myoblast culture are labelled by both anti-Thy-1 and 5.1 H11 antibodies. A few (10%) of the cells were found to be Thy-1⁺/5.1 H11⁻. These muscle cultures have been significantly depleted of fibroblasts by preplating¹⁴; we therefore believe that the Thy-1⁺/5.1 H11⁻ cells are muscle fibroblasts, because these cells express the same antigenic phenotype as the fibroblast cultures. At the mid-fusion stage of growth and differentiation (Fig. 2*d*), the mononucleate myoblast cells have started to fuse into multinucleate myotubes, although some mononucleate cells remain. All the multinucleate myotubes in the cultures are stained by 5.1 H11 antibody (Fig. 2*f*) but not by anti-Thy-1 antibodies (Fig. 2*e*). Multinucleate myotubes are therefore Thy-1⁻/5.1 H11⁺. As the myoblast population has mostly fused to form myotubes, there are few myoblasts of Thy-1⁺/5.1 H11⁺ phenotype at this time. Most of the mononucleate cells in these cultures are Thy-1⁺/5.1 H11⁻ and,

we believe, fibroblasts. At a later stage in muscle differentiation, post-fusion, the fibroblast population has enlarged and the majority of the mononucleate cells are Thy-1⁺/5.1 H11⁻. The myotubes here are still Thy-1⁻/5.1 H11⁺. In general, the strength of 5.1 H11 staining is much higher in post-fusion than in mid-fusion cultures so it is possible that the level of the antigen increases during differentiation. Two further points deserve comment. First, in mid-fusion and post-fusion cultures a small percentage of mononucleate cells is often found expressing the phenotype Thy-1⁻/5.1 H11⁺. Figure 2*j, k, l* shows an example of this behaviour and it seems likely that these cells are post-mitotic myoblasts which do not fuse to myotubes¹⁵.

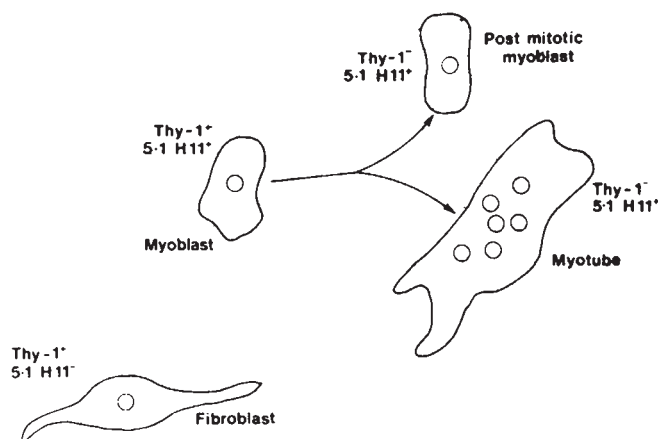


Fig. 3 A scheme for cell-surface differentiation during human myogenesis.

Second, we have confirmed the conclusions presented for the primary muscle cultures in experiments carried out on myogenic single-cell clones derived from human skeletal muscle.¹⁶ Thus, the pure populations of myoblasts in these clones are all Thy-1⁺/5.1 H11⁺ and multinucleate myotubes are Thy-1⁺/5.1 H11⁺. No Thy-1⁺/5.1 H11⁻ cells were found in either pre- or post-fusion cultures.

Thus, we have found a distinctive phenotype for each of the main cellular populations in human skeletal muscle cultures. It is therefore possible to construct a model of surface membrane changes during myogenesis incorporating these results (Fig. 3). 5.1 H11 is present throughout myogenesis, whereas Thy-1 is lost from the cell surface as myoblasts fuse to form multinucleate myotubes (a similar situation is found with rat Thy-1⁸) or when

they become post-mitotic myoblasts. Fibroblasts remain Thy-1⁺/5.1 H11⁻ throughout culture. Hence, it should be possible to separate out the various muscle cell types for biochemical analysis. A number of interesting points remain to be resolved. Exactly when is Thy-1 lost: before or after myotube formation? Could cell-surface Thy-1 be involved in the fusion process and is this differentiation pattern altered in diseases such as Duchenne muscular dystrophy? These aspects are now being investigated.

We thank Dr Roger Morris for the gift of the rabbit anti-rat Thy-1, rhodamine-conjugated sheep anti-rabbit antisera and the purified rat Thy-1 antigen, Dr Rose Yasin for the human muscle clones and Susheela Dhut for technical assistance. This work was supported by a special project grant from the Muscular Dystrophy Group of Great Britain to F.S.W.

Received 2 July; accepted 10 November 1980.

1. Pearson, M. L. in *The Molecular Genetics of Development* (eds Leighton, T. & Loomis, W. F.) (Academic, New York, in the press).
2. Merlie, J. P., Changeux, J. P. & Gros, F. *J. biol. Chem.* **253**, 2882–2891 (1978).
3. Shainberg, A. & Brik, H. *FEBS Lett.* **88**, 327–331 (1978).
4. Mir-Lechaire, F. J. & Barondes, S. H. *Nature* **272**, 256–258 (1978).
5. Podleski, T. R., Greenberg, I. & Nichols, S. C. *Exp Cell Res.* **122**, 305–316 (1979).
6. Raff, M. C., Fields, K. L., Mirsky, R., Pruss, R. M. & Winter, J. *Brain Res.* **174**, 283–308 (1979).
7. Walsh, F. S. in *Synaptic Constituents in Health and Disease* (eds Brzin, M., Sket, D. & Bachelard, H.) 285–320 (Pergamon, Oxford, 1980).

8. Lesley, J. F. & Lennon, V. A. *Nature* **268**, 163–165 (1977).
9. Stern, P. L. *Nature* **246**, 76–78 (1973).
10. Morris, R. J., Mancini, P. E. & Pfeiffer, S. E. *Brain Res.* **182**, 119–135 (1980).
11. Morris, R. J. & Ritter, M. A. *Cell Tissue Res.* **206**, 459–475 (1980).
12. Dalchau, R. & Fabre, J. W. *J. exp. Med.* **149**, 576–591 (1979).
13. Arndt, R., Stark, R., Klein, P. & Thiele, H.-G. *Immunology* **35**, 95–103 (1978).
14. Yaffe, D. in *Tissue Culture Methods and Application* (eds Kruse, P. F. & Patterson, M. K. Jr) 106–114 (Academic, New York, 1973).
15. Linkhart, T. A. & Hauschka, S. D. *Dev Biol.* **69**, 529–548 (1979).
16. Yasin, R., Kundu, D. & Thompson, E. J. *Cell Biol. int. Rep.* **4**, 783 (1980).
17. Yasin, R. *et al. J. neurol. Sci.* **32**, 347–360 (1977).

Use of a synthetic adjuvant in an effective vaccination of monkeys against malaria

Wasim A. Siddiqui, Siu-Chow Kan, Kenton Kramer, Steve Case & Kevin Palmer

Department of Tropical Medicine and Medical Microbiology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96816

John F. Niblack

Department of Pharmacology, Central Research Division, Pfizer Inc., Groton, Connecticut 06340

Research towards the development of a vaccine against malaria has recently accelerated¹, mainly because of a resurgence in the worldwide incidence of the disease². These investigations have been aided by the use of the owl monkey for direct experimentation with human malaria^{3–5}, techniques for producing *Plasmodium falciparum* antigen through continuous or short-term *in-vitro* cultivation^{6,7}, and the recent demonstration of effective immunization of owl monkeys against *P. falciparum*^{8,9}. However, the inclusion of a strong adjuvant in the vaccines seems to be required for the development of protective immunity. Thus, inclusion of the whole mycobacterial cell wall/mineral oil adjuvant (Freund's complete adjuvant: FCA)^{8,9} or a semi-synthetic derivative of the active component of that cell wall (6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine: stearoyl-MDP)^{10,11} has been necessary for the successful immunization of primates against *P. falciparum*. The use of X-ray irradiated parasites^{12–14} or substitution of FCA by muramyl-dipeptide^{15,16} does not result in significant protective immunity. Because the systemic and local toxic responses to the active adjuvants is either unacceptable (as in the case of FCA)^{10,11}, or essentially undescribed (as for stearoyl-MDP), it seemed highly desirable to attempt to identify alternative, well tolerated synthetic compounds with strong adjuvant activity for malarial vaccines. A potential candidate for this role was CP-20,961 (*N,N*-dioctadecyl-*N'*, *N'*-bis (2-hydroxyethyl)-propanediamine), a synthetic lipoidal amine, originally characterized as an interferon inducer^{17–20}, with strong adjuvant activity for humoral and cellular immune responses^{21,22}. We now report that killed *P. falciparum* merozoite antigen administered with CP-20,961 can effectively immunize owl monkeys against a lethal *P. falciparum* infection.

The adjuvant action of CP-20,961 derives from an enhancement of leukocyte traffic through antigen deposits and into the draining lymph nodes, potentiating the expansion of immunoreactive clones²³. Immunizations of guinea pigs²¹, mice²³, rats, cows and horses (unpublished data) with intramuscular vaccines adjuvanted with CP-20,961 in aqueous soybean oil emulsions result in elevated immune responses, with only mild, transient swelling at the site of injection. The nature and extent of the cellular reactions induced at the site of CP-20,961 injection and in the draining lymphatics has been well characterized and seems to be quite benign, in contrast to reactions noted with equipotent doses of FCA²³. Systemic toxicity of CP-20,961 also seems quite low when assessed either acutely (LD₅₀ > 2,000 mg per kg, mice, intraperitoneally i.p.), or chronically (unpublished data). It was these characteristics which prompted our investigation of CP-20,961 as a substitute for FCA in an experimental malaria vaccine.

The Uganda-Palo Alto strain (FUP) of *P. falciparum* has been maintained in our laboratory by serial passages of blood-induced infections in owl monkeys (*Aotus trivirgatus griseimembra*)²⁴. The immunizing antigen (mature segmenters containing individual merozoites) was obtained through short-term *in vitro* cultivation of owl monkey-derived *P. falciparum* parasites^{11,25}, and was concentrated and collected relatively free of other cellular elements²⁶. The antigen preparation was stored at –20 °C for 2 months.

CP-20,961 was prepared with carrier soybean oil emulsion (Intralipid, Cutter Laboratories) as previously described²¹. The adjuvant stearoyl-MDP was used with carrier liposomes^{10,27} and the method of malaria vaccine preparation using this adjuvant has been described earlier¹¹, as have the techniques for preparing malaria vaccine with FCA⁸.

Nine owl monkeys were used, indicated by physical characteristics and fur coloration²⁸ to be of phenotype B. Using the standard chromosome preparation method²⁹ and well established classification criteria²⁸, six monkeys (A299, A304, A300, A297, A310 and A321) were found to belong to karyotype II and the remaining three (A320, A322 & A301) to karyotype III. Monkeys of both these karyotypes, originating in Columbia, South America, were found to be highly susceptible to blood challenge from a patent *Aotus* monkey infection of the FUP strain of *P. falciparum*³⁰.

Table 1 summarizes the composition of the vaccine and the vaccination schedule. Three monkeys were used as controls: A299 and A305 received parasite antigen only and A300 CP-20,961 adjuvant only. Of the six vaccinated monkeys,

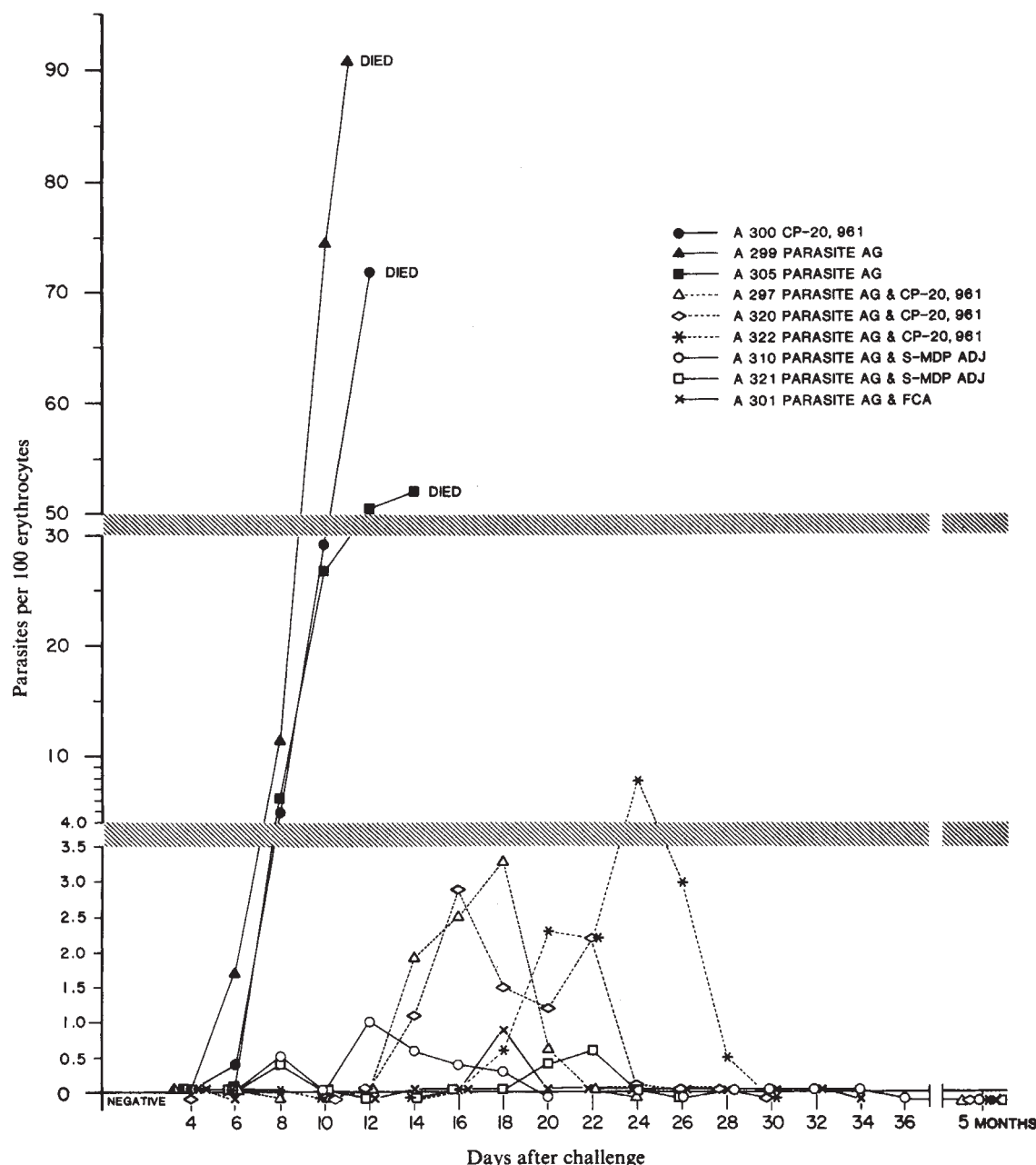


Fig. 1 Course of infection of *Plasmodium falciparum* (FUP strain) in control and vaccinated monkeys (*Aotus trivirgatus griseimembra*).

A297, A320 and A322 received vaccine composed of parasite antigen plus CP-20,961 adjuvant; A310 and A321 were vaccinated with parasite antigen mixed with stearyl-MDP; A301 received parasite antigen with FCA. Two intramuscular injections of vaccine were given in alternate thighs at a 3-week interval. Immunization never produced a detectable infection. On day 51, all nine monkeys were challenged with an intravenous injection of 7.5×10^5 parasites (FUP strain of *P. falciparum*) derived from a current infection in an owl monkey. Thick and/or thin blood films were made daily to follow the course of infection (Fig. 1). All three control monkeys died within 2 weeks of challenge: A300 and A299 died on day 12 with parasitaemias of 72.0 and 91.0%, respectively, and A305 died on day 14 with 52.2% parasitaemia. In contrast, all six vaccinated monkeys survived challenge, exhibiting only low grade infections. The duration of patency was longest in A310, which experienced a maximum parasitaemia of 1.6% and a very mild recrudescence. The duration of parasitaemia was similar in the other five vaccinated animals. Peak parasitaemias of 4%

occurred in A297 and A320 and 8% in A322, and lasted for 3 days only. In general, the level of parasitaemia remained below 0.5% in A301 and A321. Full recovery from *P. falciparum* infection began on day 24 for A297, on day 32 for A320, A321, A322 and A301, and on day 36 for A310. Although the number of monkeys used in this experiment was small, the difference between the course of infection in immunized and non-immunized monkeys is indeed very significant considering the high virulence of *P. falciparum* (FUP) for owl monkeys. The FCA-immunized monkeys displayed the usual granulomatous abscesses at the injection site, but only minimal side reactions were observed in the owl monkeys immunized with the synthetic adjuvants: stearyl-MDP induced a temporary anorexia and CP-20,961 produced a slight, transient swelling at the site of injection.

Our results confirm previous findings of protective immunity conferred by merozoite vaccines containing stearyl-MDP¹¹ and FCA^{8,9}, and further demonstrate that the novel synthetic adjuvant CP-20,961, when combined with *P. falciparum*

Table 1 Use of synthetic adjuvants in vaccination of Aotus monkeys against *Plasmodium falciparum* (Uganda-Palo Alto strain)

Monkey no.	Composition of vaccine	
	Adjuvant	Parasite antigen (1.4 mg protein per injection)
A299	None	+
A305	None	+
A300	Pfizer, CP-20,961*	None
A297	Pfizer, CP-20,961	+
A320	Pfizer, CP-20,961	+
A322	Pfizer, CP-20,961	+
A310	S-MDP†	+
A321	S-MDP	+
A301	FCA‡	+

The vaccine consisted of adjuvant plus antigen mixed using a 3-way stopcock and two syringes, administered intramuscularly in alternate thighs. Vaccination was on days 0 and 21. Parasite antigen consisted of >50% mature schizonts with individual merozoites, freed by saponin lysis, 0.5 ml per dose²¹. Challenge inoculum was with parasitized blood obtained from a current infection (FUP strain of *P. falciparum*) in an *Aotus* monkey. Inoculum was given intravenously on day 51.

* Pfizer adjuvant containing 3 mg of CP-20,961 dispersed in Intralipid in 1.0 ml volume per dose²².

† 0.25 mg of 5-O-stearoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine plus liposomes in 1.0 ml volume per dose²¹.

‡ 0.5 ml Freund's complete adjuvant (Difco) per dose.

merozoite antigen, fully protects owl monkeys against a normally lethal homologous challenge of intra-erythrocytic *P. falciparum* parasites. Although the duration of immunity conferred by malaria vaccines containing CP-20,961 or other adjuvants has not been fully assessed in the present model, antibodies against Rift Valley fever virus produced in *Macacca fascicularis* with a CP-20,961-adjuvanted vaccine persist at high levels for at least 6 months (J. A. Reynolds, personal communication).

This work was supported by AID contract ta-c-1227, UNDP/World Bank/WHO special program for research and training in tropical diseases, and Central Research Division, Pfizer Inc., USA.

Received 21 July; accepted 4 November 1980.

- WHO tech. Rep. Ser. 579 (World Health Organization, Geneva, 1975).
- Maugh, T. H. *Science* **196**, 413-416 (1977).
- Young, M. D., Porter, J. A. Jr & Johnson, C. M. *Science* **153**, 1006-1007 (1966).
- Geiman, Q. M. & Meager, M. J. *Nature* **215**, 437-439 (1967).
- Geiman, Q. M. & Siddiqui, W. A. *Am. J. trop. Med. Hyg.* **18**, 351-354 (1969).
- Trager, W. & Jensen, J. B. *Science* **193**, 673-675 (1976).
- Siddiqui, W. A., Schnell, J. V. & Richmond-Crum, S. *Am. J. trop. Med. Hyg.* **23**, 1015-1018 (1974).
- Siddiqui, W. A. *Science* **197**, 388-389 (1977).
- Mitchell, G. H., Butcher, G. A., Richards, W. H. G. & Cohen, S. *Lancet* **ii**, 1335-1338 (1977).
- Kotani, S. *et al. Biken's J.* **20**, 95-103 (1977).
- Siddiqui, W. A. *et al. Science* **201**, 1237-1239 (1978).
- Voller, A. & Richards, W. H. G. *Lancet* **ii**, 1172-1174 (1968).
- Sadun, E. H., Wellde, B. T. & Hickman, R. L. *Milit. Med.* **134**, 1165-1175 (1969).
- Wellde, B. T., Diggs, C. L. & Anderson, S. *Bull. Wild Hlth Org.* **57**, Suppl. 1, 153-157 (1979).
- Siddiqui, W. A. *et al. Bull. Wild Hlth Org.* **57**, Suppl. 1, 199-203 (1979).
- Reese, R. T., Trager, W., Jensen, J. B., Miller, D. R. & Tantravati, R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5665-5668 (1978).
- Hoffman, W. W., Korst, J. J., Niblack, J. F. & Cronin, T. H. *Antimicrob. Ag. Chemother.* **3**, 498-502 (1973).
- Glaz, E. T. & Talas, M. *Acta microbiol. acad. sci. hung.* **25**, 87-96 (1978).
- Panusarn, C., Stanley, E. D., Dirda, V., Rubenis, M. & Jackson, G. G. *New Engl. J. Med.* **291**, 57-61 (1974).
- Niblack, J. F. *Tex. Rep. Biol. Med.* **35**, 528-534 (1977).
- Niblack, J. F. *et al. J. reticuloendothel. Soc.* **S26**, 655-666 (1979).
- Chang, Y. H. & Pearson, C. M. *Arthritis Rheum.* **21**, 169-170 (1978).
- Anderson, A. O. & Reynolds, J. A. *J. reticuloendothel. Soc.* **S26**, 667-680 (1979).
- Siddiqui, W. A. & Schnell, J. V. *J. Parasit.* **59**, 516-519 (1973).
- Siddiqui, W. A. & Richmond-Crum, S. M. *J. Parasit.* **63**, 583-584 (1977).
- Siddiqui, W. A., Kramer, K. & Richmond-Crum, S. M. *J. Parasit.* **64**, 168-169 (1978).
- Inoue, K. *Biochim. biophys. Acta* **339**, 390-402 (1974).
- Ma, N. S. F., Jones, T. C., Miller, A. C., Morgan, L. M. & Adams, E. A. *Lab. Anim. Sci.* **26**, 1022-1036 (1976).
- Moorehead, B. S., Nowell, P. C., Mellman, W. J., Battips, D. M. & Hungerford, D. A. *Expl Cell Res.* **20**, 613-616 (1960).
- Taylor, W. & Siddiqui, W. A. *J. Parasit.* **65**, 267-271 (1979).

Isolation of a cloned cell line expressing variant H-2K^k using fluorescence-activated cell sorting

B. Holtkamp, M. Cramer, H. Lemke* & K. Rajewsky

Institute for Genetics, University of Cologne, Weyertal 121, D-5000 Köln 41, FRG

We describe here a method for the isolation of somatic cell variants that express a structurally altered gene product on their cell surface. The method makes it possible to select positively for cells of the variant phenotype which is defined by loss of a given antigenic determinant. As an example, we present the isolation of a cloned cell line expressing a structurally altered H-2K^k molecule, designated H-2K^{ks1}. Cells of this phenotype were found to be present in the wild-type population at a frequency of 10⁻⁵-10⁻⁶ and were isolated in three rounds of selection in the fluorescence-activated cell sorter (FACS) and subsequent growth *in vitro*.

Our method for the isolation of structural H-2K^k variants requires a set of monoclonal antibodies, each of which reacts with a single antigenic determinant on the H-2K^k molecule¹. The antigenic determinants recognized by these antibodies can be distinguished from each other by genetic analysis in which the antibodies are reacted with a set of cells expressing different H-2 haplotypes². Imagine a pair of monoclonal anti-H-2K^k antibodies, each of which binds to a different H-2K^k determinant. If one antibody is labelled with a green and the other with a red fluorescent dye and the cells stained with a mixture of the two, we could in principle directly isolate, by cell sorting, variant cells that have lost reactivity with one of the antibodies but not the other. Such cells would be expected to continue expressing an H-2K^k-like molecule, but this molecule would lack at least one antigenic determinant. Because of technical limitations of the FACS I (Becton-Dickinson), we have not yet been able to apply this simple principle directly, but have used a similar, though less general, approach (Fig. 1).

We used a pair of monoclonal antibodies which mutually inhibit their binding to the H-2K^k target, presumably by steric hindrance. Such pairs of antibodies have been shown to exist³. One of the two antibodies is labelled with a fluorescent dye and incubated with cells in the presence of a large excess of the (unlabelled) second antibody. In these conditions we expect staining of variant cells only, in particular of variants that have lost reactivity with the second antibody. Such cells can then be positively selected for in the cell sorter.

The wild-type cells used in our study were descendants of the LDHB cell line which originated from a spontaneous lymphoma of a (C3H×DBA/2)F₁ mouse and which is described in ref. 3. LDHB cells possess 41 chromosomes and carry on one of their two chromosomes no. 17 the structural gene for the H-2K^k molecule. Because H-2K^k is only poorly expressed on the surface of LDHB cells¹, a 'high-producer' subline was established by staining LDHB cells with fluorescent, monoclonal anti-H-2K^k antibodies², isolating the brightest cells in the FACS I, growing them up in culture and repeating the process. After eight cycles of sorting and subsequent growth *in vitro*, the cells were cloned by limiting dilution (cloning efficiency 70%). The cells then grew in culture for 5 months with a doubling time of 18 h, during which time they were selected for high H-2K^k expression by cell sorting five times. The resulting cell line, HK13, exhibited bright fluorescence on staining with fluorescent anti-H-2K^k antibodies, 10 times above that of spleen cells from (C3H×DBA/2)F₁ mice, and from this cell line we isolated a somatic variant with structurally altered H-2K^k.

For this purpose we used fluorescein-labelled antibody H100-5/28 for cell staining and antibody H100-27/55 as inhibitor (compare with Fig. 1). The properties of these monoclonal

* Present address: Biochemical Institute, Christian-Albrecht-University, D-2300 Kiel, FRG.

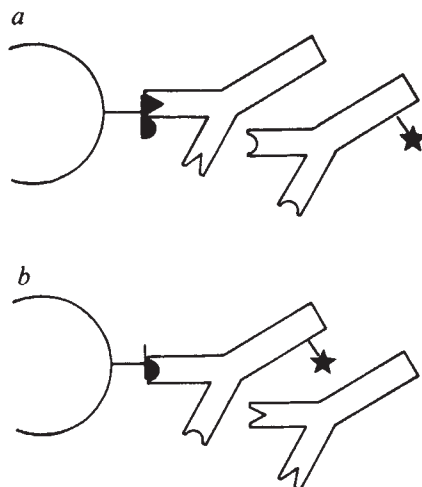


Fig. 1 Principle of variant selection. *a*, The labelling of cells carrying wild-type H-2K^k by a fluorescent (★) monoclonal antibody is inhibited by an unlabelled second antibody which binds to a neighbouring antigenic determinant. *b*, Cells expressing variant H-2K^k molecules that have lost the neighbouring determinant are successfully stained in the same experimental conditions. In reality, the unlabelled antibody is present in a 1,000-fold excess.

antibodies against H-2K^k are described in refs 1 and 2. The methods for attaching fluorescein groups to antibodies and for cell staining have been described previously³. Antibody H100-27/55 inhibited almost completely fluorescent staining of HK13 cells by antibody H100-5/28. Rare spontaneous variants whose staining could not be inhibited, and which were apparently present in a population of 2.4×10^7 HK13 cells (the total number of cells initially sorted; compare with Table 1), were isolated during three rounds of separation in the cell sorter and subsequent growth *in vitro*. The technical details of our cell-sorting procedure have been described previously⁴. Table 1 summarizes this particular series of cell sorting.

The proportion of variant (fluorescent) cells in the cell population increased dramatically after each sort (30–100-fold) and reached 78% after the third round of cell sorting and growth. These cells were again stained as before and variants were cloned directly by sorting single fluorescent cells into the wells of a 96-microwell tissue culture plate⁵. Fourteen clones were picked, reclone twice by limiting dilution (cloning efficiency ~70%) and subjected to fluorescence analysis. With one exception, all the clones behaved similarly in this analysis—almost 100% of the cells exhibited bright fluorescence when labelled with fluorescein-conjugated antibody H100-5/28, whereas the fluorescence on labelling with fluorescein-conjugated antibody H100-27/55 was close to background. (In fact the fluorescence was slightly above background. We attribute this to the slight cross-reaction of antibody H100-27/55 with the H-2D^k product² (also expressed in HK13 cells) because the staining could not be inhibited by unlabelled antibody H100-5/28.) The

binding of antibody H100-5/28 to the variant cells was not significantly inhibited by even a 1,000-fold excess of antibody H100-27/55 in a radioinhibition assay¹ (data not shown).

To demonstrate that the variant cells indeed express structurally altered H-2K^k molecules, a clone exhibiting particularly strong fluorescence with antibody H100-5/28 was selected (HK13.S1) and grown up in tissue culture. 1×10^8 variant and wild type cells (HK13) were surface iodinated with ¹²⁵I by the lactoperoxidase technique⁶ and lysed at 4 °C with 0.5% NP40 in the presence of 10^{-3} M phenylmethylsulphonyl fluoride. Nuclei were spun down and the lysate deaggregated by high-speed centrifugation. Details of the method are given in ref. 7. H-2K^k molecules were then precipitated from the lysates with monoclonal antibodies H100-27/55 and H100-5/28 and analysed on SDS-polyacrylamide gels (for experimental details see Fig. 2 legend). The results are shown in Fig. 2.

In the case of the HK13 cell lysate, the familiar 45,000- and 12,000-molecular weight bands of H-2K heavy and light chains appear on the gels on precipitation with either of the two monoclonal antibodies (tracks *a* and *b*). As staining of HK13 cells by either antibody can be completely inhibited by the other, we assume that the protein bands in tracks *a* and *b* represent the heavy and light chains of a single H-2K^k molecule that co-expresses the determinants recognized by the two antibodies. With the variant cell lysate, bands of similar size appear on the gel, but only on precipitation with antibody H100-5/28; anti-

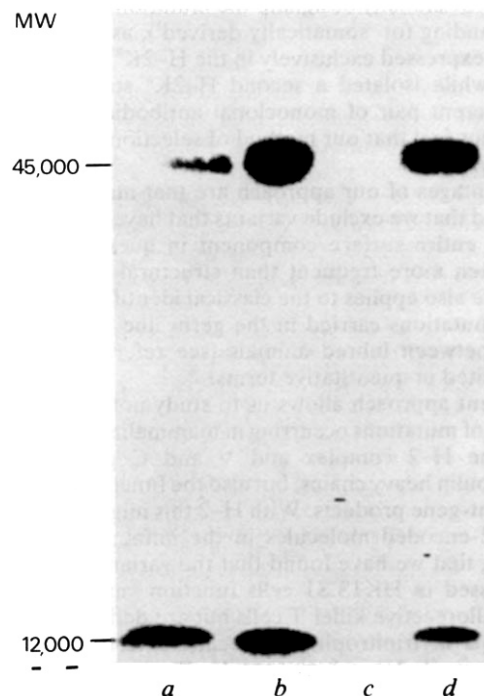


Fig. 2 Electrophoretic analysis of precipitations of wild-type and variant H-2K^k molecules with monoclonal antibodies H100-27/55 and H100-5/28. Detergent lysates (equivalent to 10^7 surface-iodinated cells) were incubated with 30 µg antibody (DEAE-cellulose purified) at 4 °C for 6 h and the immunoglobulin (IgG2a in both cases) was bound to 25 µl protein A-Sepharose (Pharmacia). After appropriate washings; sample buffer 4% SDS, 6 M urea, 10% 2-mercaptoethanol and bromophenol blue (tracking dye) was used to dissociate the antigen-antibody-protein A-Sepharose complexes. The figure shows the autoradiograph (8 days exposure) of a 10–15% gradient polyacrylamide slab gel run in 0.1% SDS in the discontinuous system of Laemmli¹⁰. One aliquot of HK13 (wild-type) lysate was precipitated with antibody H100-27/55 (track *a*) and another aliquot was precipitated with antibody H100-5/28 (track *b*). Similarly, HK13.S1 lysate (variant type) was precipitated with antibody H100-27/55 (track *c*) and with antibody H100-5/28 (track *d*). As identical amounts of antibodies H100-27/55 and H100-5/28 were used in our precipitating procedure, we consider the fainter bands in track *a* to be due to a lower precipitation capacity of antibody H100-27/55. MW, molecular weight.

Table 1 Protocol of H-2K^k variant enrichment

Sorting*	No. of cells sorted	% Cells deflected	% Fluorescent cells†
1st	2.4×10^7	1.2	0.025 (2×10^4)‡
2nd	2.9×10^7	2.0	0.65 (2.3×10^3)‡
3rd	2.1×10^7	0.6	77.5 (4×10^4)§

* Cells were incubated with 0.02 mg ml^{-1} fluorescein-labelled monoclonal antibody H100-5/28 and a 10^3 -fold excess of unlabelled monoclonal antibody H100-27/55. Fluorescent cells were separated in the FACS.

† After sorting and subsequent growth *in vitro*. Values in parentheses indicate total number of cells analysed.

‡ Determined by microscopic analysis.

§ Determined by cell-sorter analysis.

body H100-27/55 does not bring down any detectable radioactivity (tracks *c* and *d*). Taken together, the data show that HK13.S1 cell membranes contain variant H-2K^k molecules, whose polypeptide chains are of similar size to those of the wild-type molecules but which lack one of the two antigenic determinants recognized by our two monoclonal antibodies and are co-expressed in wild-type H-2K^k. The different fluorescent-staining patterns of wild-type and variant cells are thus due to an alteration of H-2K^k itself and not, for example, to a change in its orientation in the cell membrane.

The selection method described here makes it possible to isolate efficiently and at will a somatic-cell variant that stably expresses structurally altered H-2K^k molecules on the surface. From the data in Table 1, we conclude that spontaneous variants of the selected phenotype were present in the population of HK13 cells at a frequency of 10^{-5} – 10^{-6} . Taking into account the generation time of the cells and the number of generations after cloning, we estimated roughly a frequency of variation of 10^{-6} – 10^{-7} per cell per generation. This low frequency and the stability of the variant suggest that the phenotypic change in HK13.S1 cells is due to mutation. Preliminary experiments indicate that both antibodies used in the variant selection also react with unglycosylated H-2K^k molecules. The mutation carried by HK13.S1 cells may therefore have occurred in the structural gene for H-2K^k. Structural analysis of the variant protein or of the corresponding genetic material is required to establish this point. We provisionally designate the mutation as H-2K^{ks1} (the prefix 's' standing for 'somatically derived'), assuming that the mutation is expressed exclusively in the H-2K^{ks1} molecule. We have meanwhile isolated a second H-2K^k structural variant using a different pair of monoclonal antibodies (unpublished data) and thus feel that our method of selection may have wide applicability.

The advantages of our approach are that many cells can be screened and that we exclude variants that have lost the expression of the entire surface component in question—an event which is often more frequent than structural variation⁸. The latter feature also applies to the classical identification of structural H-2 mutations carried in the germ line by tissue transplantation between inbred animals (see ref. 9) but which is severely limited in quantitative terms.

The present approach allows us to study not only types and frequencies of mutations occurring in mammalian genes, such as genes in the H-2 complex and V and C genes encoding immunoglobulin heavy chains, but also the functional properties of the variant-gene products. With H-2 this might elucidate the role of H-2-encoded molecules in the immune system. It is encouraging that we have found that the variant H-2K^k molecules expressed in HK13.S1 cells function very efficiently as targets for alloreactive killer T cells but are deficient as restricting elements in trinitrophenyl-specific, H-2-restricted cytotoxic reactions (J. Neuerburg and H.-W. Vohr, unpublished data).

This work was supported by the Deutsche Forschungsgemeinschaft through SFB 74 and the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen. We thank W. Santüns for help in the cell sorter experiments and Ms C. Schenkel for technical assistance, Dr K. Reske for technical advice and Dr A. Radbruch for analysing the LDHB cell karyotype.

Received 2 September; accepted 7 November 1980.

1. Lemke, H., Hämmerling, G. J. & Hämmerling, U. *Immun. Rev.* **47**, 175–206 (1979).
2. Klein, J., Huang, H. S., Lemke, H., Hämmerling, G. J. & Hämmerling, U. *Immunogenetics* **8**, 419–432 (1979).
3. Holtkamp, B., Lindahl, K. F., Segall, M. & Rajewsky, K. *Immunogenetics* **9**, 405–421 (1979).
4. Liesegang, B., Radbruch, A. & Rajewsky, K. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3901–3905 (1978).
5. Parks, D. R., Bryan, V. M., Oi, V. T. & Herzenberg, L. A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1962–1966 (1979).
6. Hausteint, D. J. *Immun. Meth.* **27**, 25–38 (1975).
7. Jones, P. P. *J. exp. Med.* **146**, 1261–1279 (1977).
8. Rajan, T. V. *Immunogenetics* **10**, 423–431 (1980).
9. Klein, J. *Adv. Immun.* **26**, 55–146 (1978).
10. Laemmli, U. K. *Nature* **227**, 680–685 (1970).

Expression of cell-surface HLA-DR, HLA-ABC and glycophorin during erythroid differentiation

Jean Robinson*, Colin Sieff†, Domenico Delia*, Paul A. W. Edwards‡ & Melvyn Greaves*

* Membrane Immunology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK

† Department of Haematology, The Hospital for Sick Children, Great Ormond Street, London WC1, UK

‡ Ludwig Institute for Cancer Research, Sutton, Surrey SM2 5PX, UK

The unexpected discovery that Ia-like (HLA-DR) antigens^{1–3} in humans were present on blast cells from acute myeloblastic leukaemia^{4,5} led to the finding that normal granulocytic progenitors, in contrast to their mature descendants, also expressed HLA-DR antigens. Thus, anti-Ia sera stain a proportion of myeloblasts in normal bone marrow^{6,7}, inhibit myeloid progenitor (CFU-GM) colony formation in the presence of complement⁸ and can be used to label and separate CFU-GM on a fluorescence-activated cell sorter (FACS)⁹. Winchester *et al.* subsequently reported that erythroid progenitors (BFU-E and CFU-E) were also inhibited or killed by anti-Ia (p28,37) and complement¹⁰. These observations raised the possibility that HLA-DR (or presumptive I-region equivalent) products might have a regulatory role in early haematopoiesis. We have now analysed HLA-DR and HLA-ABC antigen expression on normal erythroid progenitors using monoclonal antibodies to non-polymorphic determinants and fluorescence-activated cell sorting. In parallel experiments, we tested a monoclonal antibody to glycophorin, a well defined erythroid-specific cell-surface membrane glycoprotein¹¹. We report that HLA-DR, HLA-ABC and glycophorin are all expressed at various stages during erythroid differentiation.

Table 1 lists the results of assays for erythroid burst-forming units (BFU-E) and colony-forming units (CFU-E) in a series of experiments in which monoclonal anti-HLA-DR was used to stain blood or marrow cells followed by analysis and sorting on the FACS (Fig. 1).

The majority of recoverable BFU-E consistently seem to localize in the HLA-DR-positive population, whereas CFU-E are found mostly, but not exclusively, in the fraction which has either low density or no HLA-DR. When light scattering was used as an additional parameter for sorting, both the BFU-E and CFU-E seemed to be restricted to the population within the larger light-scattering (or presumed bigger) cell size range.

The total recovery of BFU-E in the separated fractions was, however, only approximately one-third (Table 1 legend). Much higher recovery of CFU-E in these experiments and of BFU-E in experiments with anti-HLA-ABC and anti-glycophorin indicate that this is probably not a technical artefact; neither is it attributable to an inhibiting effect of the monoclonal anti-HLA-DR because no diminution of BFU-E activity is seen in the stained but unsorted cells (see control 2 in Table 1). This result may therefore indicate that sorting on the basis of HLA-DR expression separates cells whose interaction may be required for optimal BFU-E activity.

When monoclonal anti-HLA-ABC (PA 2.6) was used to stain bone marrow cells, all BFU-E and CFU-E were sorted into the HLA-ABC⁺ (positive) population (Table 1, experiments 6, 7). Bone marrow cells were sorted on the basis of intensity of fluorescence staining with monoclonal anti-glycophorin (Fig. 1). More than 99% of both BFU-E and CFU-E were found in the fraction giving minimal or negative staining (Table 1, experiments 8, 9).

Less than 5.0% of the morphologically recognizable erythroid cells were separated into the HLA-DR-positive fractions. By contrast, almost all the cells in the glycophorin-positive popu-

Table 1 Separation of BFU-E and CFU-E with monoclonal antibody and the FACS

Expt	Antibody	Fraction	% Cells	CFU-E			Colony assays		BFU-E	
				Per 10 ⁵ cells	Recovery per 10 ⁵ unfractionated cells (%)	% Of total recovered†	Per 10 ⁵ cells	Recovery per 10 ⁵ unfractionated cells (%)	% Of total recovered	
1	DA2 (HLA-DR)	1. Control					23			
		2. Control					18			
		Sorted:								
		3. +Large } 4. +Small }	30				14 } 149 }	24.5	95.3	
		5. Negative	60				2	1.2	4.7	
							25.7(125)*			
2	DA2 (HLA-DR)	1. Control					15.8			
		2. Control					8.3			
		Sorted:								
		3. +Large } 4. +Small }	10				19.4	1.94	100	
		5. Negative	6.5				0	0	0	
							0			
3	DA2 (HLA-DR)	1. Control		38.8			12.5			
		2. Control		46.8			14.3			
		Sorted:								
		3. +Large } 4. +Small }	3	288	8.6	24	133	3.99	99.5	
		5. Negative	1	33	0.3	0.9	2	0.02	0.5	
							0			
4	DA2 (HLA-DR)	1. Control								
		2. Control								
		Sorted:								
		3. +Large } 4. +Small }	9	94	8.5	14.9	56	5.04	89.4	
		5. Negative	10	10.5	1.0	1.8	1	0.14	1.8	
							8.8			
5	DA2 (HLA-DR)	1. Control								
		2. Control								
		Sorted:								
		3. +Bright } 4. +Dim }	9	94	8.5	3.3	109	9.8	16.0	
		5. Negative	10	676	67.6	26.0	400.5	40.0	65.3	
							18.7			
6	PA 2.6 (HLA-ABC)	1. Control		210						
		2. Control		302						
		Sorted:								
		3. +Bright/dim } 4. Negative }	42	258	108	92	121	50.8	93	
			32	27.5	8.9	8	12	3.8	7	
							117 (45)			
7	PA 2.6 (HLA-ABC)	1. Control		312						
		2. Control		313						
		Sorted:								
		3. +Bright/dim } 4. Negative }	61	410	250.0	97.3	199	121.4	99.6	
			22	32	7.05	2.7	2	0.4	0.3	
							257.05 (82)			
8	28/10.1 (Glycophorin)	1. Control		112.5						
		2. Control		123.5						
		Sorted:								
		3. +Bright } 4. +Dim }	3.4	44.4 } 40.6 }	1.4	1.0	35.6 } 26.3 }	1.05	1.1	
		5. Negative	96.6	150	144.9	99	100.5	97.1	98.9	
							146.3 (123)			
9	28/10.1 (Glycophorin)	1. Control		371						
		2. Control		321						
		Sorted:								
		3. +Bright } 4. +Dim }	5	31.4	1.6	0.5	2.8	0.14	0.14	
		5. Negative	8	98.5	7.9	2.5	15.5	1.24	1.26	
							98.6			
							321 (93)			
							97.7(79)			

The unfractionated controls and the positive and negative cell fractions were washed three times in cold alpha medium (Flow) and 2% fetal calf serum (FCS). The cells were then cultured in a mixture containing 30% FCS (Gibco Bio-Cult), 1% bovine serum albumin (Sigma), 10^{-4} M mercaptoethanol, penicillin, streptomycin, 0.9% methylcellulose (Dow) and 2.5 units ml^{-1} erythropoietin (Connaught Step III)²⁴. The final concentration of nucleated cells was 1 or $2 \times 10^5 \text{ ml}^{-1}$, although occasionally $< 1 \times 10^5$ cells were obtained for some of the fractions. Volumes of 1 ml of the mixture were plated in 35-mm tissue culture plates (Nunc) in duplicate, and incubated at 37°C in a high humidity 5% $\text{CO}_2/95\%$ air tissue-culture incubator. Recognizable CFU-E, identified by the pink-red colour of the cells and comprising one or two clusters, were counted directly on day 7 using an inverted microscope at $\times 40$ magnification. BFU-E, large, red colonies comprising three or more sub-colonies, were counted on day 14. In each experiment, controls 1 and 2 are unlabelled and labelled fractionated cells, respectively. Recovery per 10^5 unfractionated cells is determined by multiplying the number of colonies per 10^5 cells (column 2) by the proportion of cells in that fraction.

* Total colonies recovered, expressed (in parentheses) as a percentage of the mean of the two unfractionated controls. Note, however, that selection of cells on the FACS involves some loss of cells.

† Number of colonies recovered in different fractions expressed as a percentage of total colonies recovered.

lation were identifiable as erythroid precursors at different stages of maturation; almost no recognizable erythroid cells were found in the glycophorin-negative fraction.

It has been suggested that BFU-E activity is enhanced by T cells¹². As most T cells have no detectable glycophorin and are

largely though not entirely HLA-DR negative, potential T-cell-BFU-E interactions could be compromised by the cell-separation experiments described above. To investigate this possibility we labelled bone marrow T cells with a mixture of two pan-T monoclonal antibodies OKT1 (ref. 13) and OKT11 (ref. 14),

separated stained and unstained cells on the FACS and assessed the effect of recombination of these fractions. The results (Table 2) indicate that effectively all BFU-E and CFU-E are unreactive with these particular monoclonal anti-T reagents and that bone marrow T cells have, if anything, an inhibitory effect on BFU-E at the cell concentrations used. Further experiments are required to investigate this possibility more thoroughly. Note, however, that the reported promoting effect of blood T cells on BFU-E may be restricted to circulating erythroid progenitors¹² and that this response has not been confirmed in another laboratory¹⁵.

Fluorescence-activated cell sorting with monoclonal antibodies provides an incisive approach to the analysis of cell-surface antigen expression on numerically infrequent progenitor cells. Our data using this approach suggest that the earliest detectable, committed erythroid progenitors, BFU-E, express HLA-DR as well as HLA-ABC antigens and that the former are progressively lost during differentiation through CFU-E to morphologically recognizable erythroblasts. HLA-ABC antigens are lost more slowly, for the majority of CFU-E are positive, as are at least a proportion of morphologically recognizable precursors. Although mature red blood cells have no detectable HLA-ABC, previous studies have indicated that normoblasts and reticulocytes may express at least a low density of these antigens¹⁶⁻¹⁸.

In contrast, the antigenic determinant of glycophorin detected by the monoclonal antibody we have used is not expressed until the post-CFU-E, HLA-DR-negative stage of erythroid maturation. More than 90% of all morphologically recognizable erythroblasts were sorted into the glycophorin⁺, HLA-DR⁻ fraction on the FACS. Gahmberg *et al.* similarly reported that basophilic normoblasts had a low density of cell-surface glycophorin but could not see binding of rabbit antibodies to glycophorin on less mature pronormoblasts¹⁹.

We incidentally noted that CFU-GM (which grow in colony assays for erythroid cells) separate predominantly in the HLA-DR⁺, HLA-ABC⁺, glycophorin⁻ fractions. This confirms previous cell-sorting experiments in which CFU-GM were present in the fraction stained with non-monoclonal anti-p28,33 (Ia-like or HLA-DR antigen)⁹.

Table 2 Effect of bone marrow T cells on BFU-E and CFU-E

Expt	Fraction	CFU-E per 10 ⁵ cells			BFU-E per 10 ⁵ cells		
		Obs.	Exp.*	Obs./Exp.	Obs.	Exp.	Obs./Exp.
(1)	1. Control	182			72		
	Sorted:						
	2. Negative	406			266		
	3. Bright ⁺	4.5			2.5		
	4. Dim ⁺	36.5			21.7		
	Sorted and recombined:						
(2)	5. Negative + bright ⁺	148.5	132	1.1	47.5	86.3	0.55
	6. Negative + dim ⁺	154	148	1.0	99.5	96.3	1.0
	1. Control	415			144		
	Sorted:						
	2. Negative	548			302.5		
	3. Bright ⁺	66			12.5		
	4. Dim ⁺	104			29.5		
	Sorted and recombined:						
	5. Negative + bright ⁺	330	299	1.1	123.5	156	0.8
	6. Negative + dim ⁺	331	326	1.0	115	166	0.7

Bone marrow cells stained with optimal concentration of OKT1 plus OKT11, sorted on the FACS and plated *in vitro* for CFU-E and BFU-E assays (see Table 1 legend). Control = stained but unsorted. Expt 1.5: negative (non-T)/bright (T) ratio 1:1. Expt 1.6: negative (non-T)/dim (T) ratio 0.6:1 (insufficient cells available for 1:1 ratio). Expt 2.5: negative (non-T)/bright (T) ratio 1:0.8 (insufficient cells available for 1:1 ratio). Expt 2.6: negative (non-T)/dim (T) ratio 1:1.

* Calculated for the number of cells added to mixtures and the ratio of CFU-E to BFU-E per 10⁵ cells in each separated population before mixing.

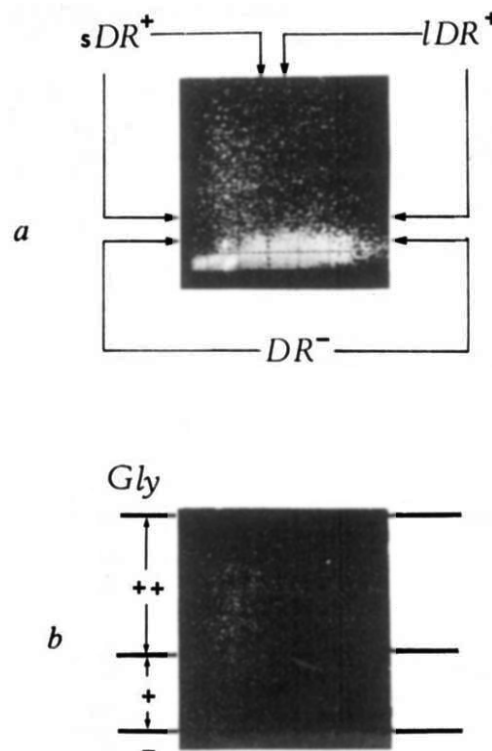


Fig. 1 Separation of erythroid precursors using monoclonal anti-HLA-DR and the FACS. Normal bone marrow was obtained by aspiration from the posterior iliac crest of adult human volunteers, or during femoral osteotomy of haematologically normal children. Approximately 5–10 ml of marrow from two to four aspirations were collected into 10 ml Hank's buffered salt solution containing 100 U of preservative-free heparin. Marrow suspensions were centrifuged (400g for 40 min at 20°C), in Ficoll-Isopaque (1.077 g cm⁻³), the interface mononuclear cells collected, washed twice and suspended in Eagle's medium plus 20% FCS. Murine monoclonal antibodies were produced using cell hybridization methods as described previously. Monoclonal anti-HLA-DR (DA2) identifies a non-polymorphic determinant associated with an Ia-like polypeptide (p28,34) complex on B lymphoblasts²². Monoclonal antibody to HLA-ABC (PA 2.6) similarly recognizes a non-polymorphic determinant shared by products of the A, B, C but not the D locus²². Monoclonal antibody LICR.LON/R10 was raised against erythrocytes and specifically precipitates glycophorin A²³. 20–100 × 10⁶ bone marrow cells in 50–100 µl were incubated for 30 min at 4°C with previously determined optimal concentrations of the various monoclonal antibodies. After two washes, the cells were stained with an affinity-purified F(ab')₂ preparation of goat antibodies which had been cross-absorbed with insolubilized human immunoglobulin and labelled with fluorescein isothiocyanate. The stained cell suspension was analysed by FACS (modified FACS-I, Becton Dickinson)⁹. Cells were separated in sterile conditions using relative fluorescence intensity (vertical axis, a, b) and in some experiments relative light-scattering levels (horizontal axis) to separate cells according to size (b) (sDR⁺ = small HLA-DR-positive cells; lDR⁺ = large HLA-DR-positive cells; DR⁻ = total HLA-DR-negative cells; Gly = glycophorin; ++, brightly stained with anti-glycophorin; +, dimly stained; -, unstained).

Winchester *et al.*¹⁰ previously reported that both BFU-E and CFU-E could be inhibited by treatment with a rabbit anti-p28,37 (Ia-like) serum and rabbit complement before colony assay. This observation suggests that erythroid progenitors express Ia-like (HLA-DR) cell-surface antigens and/or that other cells essential for BFU-E and CFU-E activity are inhibited by the antisera used. The experiments reported above demonstrate unequivocally that some BFU-E express a monomorphic HLA-DR antigenic determinant but that CFU-E were predominantly HLA-DR negative. Anti-p28,33 (Ia-like) antisera may have exceedingly high cytotoxic titres which are in excess of binding titres as assessed by immunofluorescence¹¹. It is therefore possible that CFU-E have a very low density of HLA-DR antigens which was below our 'selected' level of positivity on the FACS but sufficient to render the cells susceptible to antibody-dependent lysis (or functional inhibition) by complement.

Pluripotent haematopoietic stem cells seem to be Ia-antigen (HLA-DR equivalent) negative in mice²⁰, and possibly also in man²¹. The transient expression of HLA-DR on early erythroid

and granulocytic lineage progenitors suggests that cell-surface molecules carrying these determinants might be important in cell interactions regulating committed progenitor cells. Note, finally, that as there is no evidence for biosynthesis of HLA-DR by these progenitor cells, we cannot exclude the possibility that these cell-surface-associated molecules are somehow 'acquired' from other cells in the bone marrow.

This work was supported in part by the Imperial Cancer Research Fund and Child Health Research Appeal Trust. We thank Dr W. Bodmer for supplying monoclonal antibodies to HLA-DR and HLA-ABC and Drs G. Goldstein and P. Kung (Ortho Pharmaceuticals) for monoclonal anti-T reagents (OKT1, OKT11).

Received 4 July; accepted 21 October 1980.

1. Moller, G. *Transplant Rev.* **30**, (1976).
2. Allison, J. P. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3952 (1978).
3. Barnstable, C. J. *et al. Cold Spring Harb. Symp. quant. Biol.* **41**, 443 (1977).
4. Schlossman, S. F., Chess, L., Humphreys, R. E. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1288 (1976).
5. Greaves, M. F. & Janosy, G. *Biochim. biophys. Acta* **516**, 193 (1978).
6. Winchester, R. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 4012 (1977).
7. Janosy, G. *et al. Br. J. Haemat.* **37**, 391 (1977).
8. Koefler, H. P., Niskanen, E., Cline, M., Billing, R. & Golde, D. *Blood* **54**, 1188 (1979).
9. Janosy, G., Francis, G., Capellaro, D., Goldstone, A. & Greaves, M. F. *Nature* **276**, 176 (1978).
10. Winchester, R. J. *et al. J. exp. Med.* **148**, 613 (1978).
11. Marchesi, V. T., Furthmayr, H. & Tomita, M. *A. Rev. Biochem.* **45**, 667 (1976).
12. Nathan, D. *et al. J. exp. Med.* **147**, 324 (1978).
13. Kung, P. C., Goldstein, G., Reinherz, E. L. & Schlossman, S. F. *Science* **206**, 347 (1979).
14. Greaves, M. F. *et al. in Modern Trends in Human Leukaemia IV* (eds Neth, R., Gallo, R. C., Hofschneider, P.-H. & Mannweiler, K.) (Springer, in the press).
15. Nomdedeu, B. *et al. Expl Hemat.* **8**, 854 (1980).
16. Brown, G., Biberfeld, P., Christensson, B. & Mason, D. Y. *Eur. J. Immun.* **9**, 272 (1979).
17. Kourilsky, F. M. *et al. J. Immun.* **106**, 454 (1971).
18. Harris, R. & Zervas, J. D. *Nature* **221**, 1062 (1969).
19. Gahmberg, C. G., Jokinen, M. & Andersson, L. C. *Blood* **52**, 379 (1978).
20. Basch, R. S., Janosy, G. & Greaves, M. F. *Nature* **270**, 520 (1977).
21. Moore, M. A. S. *et al. Blood* **55**, 682 (1980).
22. Brodsky, F. M., Parham, P., Barnstable, C. J., Crumpton, M. J. & Bodmer, W. F. *Immun. Rev.* **47**, 3 (1979).
23. Edwards, P. A. W. *Biochem. Soc. Trans.* **8**, 334 (1980).
24. Iscove, N. N. & Sieber, F. *Expl Hemat.* **3**, 32 (1975).

Tetrapeptides of the eosinophil chemotactic factor of anaphylaxis (ECF-A) enhance eosinophil Fc receptor

M. Capron, A. Capron, E. J. Goetzl & K. F. Austen

Centre d'Immunologie et de Biologie Parasitaire, Institut Pasteur, 59019 Lille Cedex, France
Howard Hughes Medical Institute Laboratory at Harvard Medical School, Boston, Massachusetts 02115
Robert B. Brigham Hospital, Harvard Medical School, Seeley G. Mudd Bldg, 250 Longwood Ave, Boston, Massachusetts 02115

Certain mast cell products, including the tetrapeptides Ala-Gly-Ser-Glu (Ala⁴) and Val-Gly-Ser-Glu (Val⁴) of the eosinophil chemotactic factor of anaphylaxis (ECF-A)¹ and histamine², are preferentially chemotactic for eosinophils *in vitro*. Amino acid substitutions or modifications of the ECF-A tetrapeptides may substantially alter the chemotactic activity of human eosinophils³. The cellular mechanism whereby ECF-A tetrapeptides enhance IgG-dependent killing of schistosomula by rat eosinophils⁴ is, however, unclear. We now report that the parent tetrapeptides and the substituted analogue Val-Pro-Ser-Glu (Pro³), but not histamine, increase the number of rat and human eosinophils that form rosettes with erythrocytes bearing IgG antibodies. Moreover, we find a correlation between the effect of these substances on the expression of rat eosinophil IgG Fc receptors and their capacity to increase the IgG-dependent cytotoxicity of rat eosinophils for *Schistosoma mansoni* schistosomula.

Rat eosinophil-rich populations were composed of non-adherent peritoneal cells from normal Fischer rats⁴. Rat peritoneal neutrophils were obtained 7 h after the intraperitoneal

injection of 10% proteose-peptone in saline. Human eosinophils and neutrophils were obtained from venous blood of normal subjects or patients with hypereosinophilia not due to parasitic infections and prepared by centrifugation on metrizamide discontinuous gradients⁵. The different leukocyte populations were adjusted to $4 \times 10^6 \text{ ml}^{-1}$ in minimal essential medium. Various concentrations of the agonists or medium alone were added to the leukocyte populations and the mixtures were incubated under a CO₂ atmosphere at 37 °C with constant agitation. Immunoglobulin Fc receptors were estimated by the EA-rosette technique using sheep erythrocytes (E) sensitized with the IgG fraction of antisera to E prepared in rats (EA rat) or in rabbits (EA rabbit)⁶ (Fig. 1).

The percentage of control rat eosinophils that formed EA rosettes was significantly higher with EA rabbit than with EA rat (Fig. 1), perhaps implying a higher affinity of the rat eosinophil Fc receptor for rabbit immunoglobulins. (Similar differences with immunoglobulins from different animal species have already been noticed with guinea pig eosinophils⁷.) When rat eosinophils were pretreated with increasing concentrations of Val⁴, Ala⁴ and Pro³ tetrapeptide, there was a dose-dependent increase in the percentage of eosinophils that formed EA rat rosettes. Neither Val-Gly-Ser-Glu-methyl ester nor the lower concentrations of histamine significantly altered the expression of rat IgG Fc receptors on eosinophils, but 10^{-4} M histamine depressed receptor expression (Fig. 1a). The lowest concentration of three of the peptides and all concentrations of the Val-Gly-Ser-Glu-methyl ester and histamine examined suppressed the expression of receptors for EA rabbit relative to

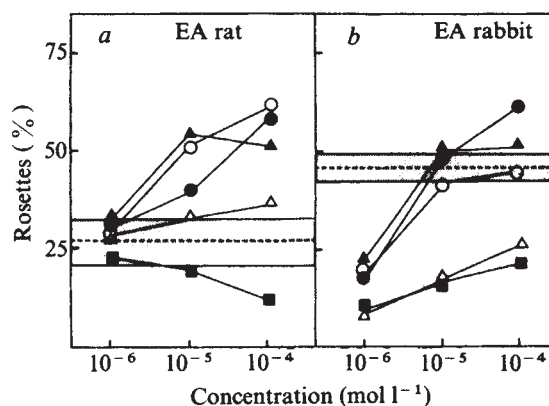


Fig. 1 Role of increasing concentrations of the ECF-A tetrapeptides Ala-Gly-Ser-Glu (●) (Serva) and Val-Gly-Ser-Glu (○), the analogues Val-Pro-Ser-Glu (▲) and Val-Gly-Ser-Glu-methyl ester (Δ) and histamine (■) (Sigma) on EA rat and EA rabbit rosette formation by rat eosinophils after 60 min incubation at 37 °C. A 60-min incubation was used for all subsequent experiments, as a preliminary study on the influence of incubation time has shown that the maximum effect occurs after 60 min incubation. Abscissa: final concentration of the products. The dotted line represents the mean percentage rosettes of rat eosinophils incubated for 60 min with minimal essential medium ($\pm$ s.e.). The γ -globulin (IgG) fraction of rat or rabbit antisera to sheep erythrocytes (E) was prepared as described in ref. 6. Rat IgG-coated E (EA rat) or rabbit IgG-coated E (EA rabbit) were prepared by mixing equal volumes of rat or rabbit IgG anti-E with a 2% solution of fresh E as described⁶. Mixtures of 0.1 ml of the leukocyte suspensions and 0.1 ml of E, EA rat or EA rabbit at a concentration of $3 \times 10^8 \text{ ml}^{-1}$ were centrifuged at 100g for 10 min at 4 °C and incubated for 30 min at 0 °C. The pellet was gently resuspended and cytofluorimetric preparations were made. These were fixed in methanol and treated with Giemsa stain. All leukocytes with three or more adherent EA were considered to be rosettes. The percentage of 300 eosinophils that formed rosettes was counted for each slide. All results are expressed as the mean of the values for triplicate studies. Val-Gly-Ser-Glu, Val-Pro-Ser-Glu and Val-Gly-Ser-Glu-methyl ester were prepared according to ref. 3. Highly purified peptides were prepared by standard solid phase method and subsequently purified by sequential application of gel filtration on Sephadex G-25 in 0.05 M acetic acid and then reverse phase HPLC on a C18 column using sodium phosphate 0.02 M, pH 4.2. Thus, no active fragment of endotoxin could be present. Each point represents the mean of at least five experiments. Data were compared by one-way analysis of variance ($P < 0.05$) and Student's *t*-test. The increase in EA rat rosettes was significant for Ala-Gly-Ser-Glu ($10^{-4} \text{ mol l}^{-1}$) and Val-Gly-Ser-Glu and Val-Pro-Ser-Glu (10^{-4} and $10^{-5} \text{ mol l}^{-1}$) ($P < 0.05$, a). Ala-Gly-Ser-Glu significantly increased the % of EA rabbit rosettes at the $10^{-4} \text{ mol l}^{-1}$ concentration ($P < 0.05$, b).

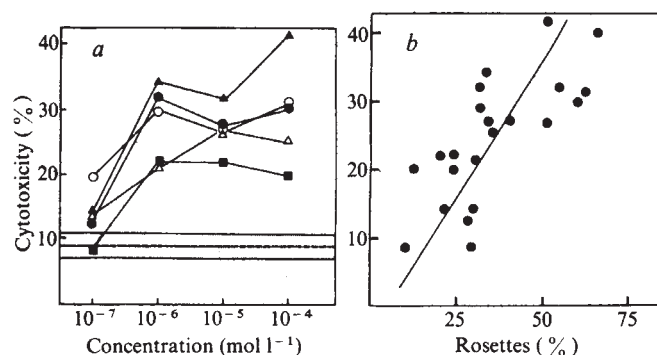


Fig. 2 a, Effect of increasing concentrations of Ala-Gly-Ser-Glu (●), Val-Gly-Ser-Glu (○), Val-Pro-Ser-Glu (▲), Val-Gly-Ser-Glu-methyl ester (△) and histamine (■) on rat IgG-dependent eosinophil cytotoxicity for schistosomula *in vitro*. Abscissa: final concentration of the products in the cytotoxicity assay. Ordinate: percentage dead schistosomula after 48 h contact with effector cells. *S. mansoni* schistosomula (prepared by penetration of cercariae through mice abdominal skin) were incubated overnight with heat-inactivated sera from *S. mansoni* infected rats⁴. Eosinophil-rich cell populations that were initially composed of 49% eosinophils and 10% mast cells were depleted of mast cells by centrifugation on 22.5% metrizamide¹⁴ to yield a population of 53% eosinophils and 0.5% mast cells. The purified eosinophils were added to antibody-coated schistosomula at an effector to target ratio of 6,000:1, in a total volume of 200 μ l. Aliquots (20 μ l) of the various mediators were then added to each well of the cytotoxicity assay, giving final concentrations of 10⁻⁷–10⁻⁴ mol l⁻¹ (ref. 4). All values represent the mean of 3–13 experiments. Data were compared by one way analysis of variance ($P < 0.01$) and Student's *t*-test. The percentage of cytotoxicity was measured by direct visual examination of dead schistosomula in triplicate experiments⁴. The dotted line represents the mean percentage of cytotoxicity $\pm$ s.e. of antibody-coated schistosomula incubated with the mast cell-depleted eosinophils in medium alone. Ala-Gly-Ser-Glu, Val-Gly-Ser-Glu and Val-Pro-Ser-Glu significantly increased the levels of cytotoxicity for the three highest concentrations ($P < 0.05$). The increase with Val-Gly-Ser-Glu-methyl ester is only significant for the 10⁻⁵ mol l⁻¹ concentration, and no significant effect was obtained with histamine. b, Correlation between the percentage of rat eosinophils that formed EA rat rosettes (abscissa) and the IgG-dependent cytotoxicity of rat eosinophils for schistosomula (ordinate), in the presence of the different mediators that had been tested (see Fig. 1a and a above). The effect of different concentrations of the mediators on the expression of rat IgG Fc receptors is significantly correlated with the increase in IgG-dependent rat eosinophil cytotoxicity for *S. mansoni* schistosomula ($r = 0.72$, $P < 0.001$; $n = 23$). A statistical analysis showed best fitting with a first-order regression line.

the levels detected on control eosinophils. Only 10⁻⁴ M Ala-Gly-Ser-Glu significantly increased the apparent number of EA rabbit rosettes (Fig. 1b).

The experimental model previously used to show that the ECF-A tetrapeptides could stimulate rat IgG-dependent eosinophil cytotoxicity for *S. mansoni* schistosomula *in vitro*⁴ was applied in parallel with the EA-rosette assay. A population of normal rat peritoneal cells enriched in eosinophils and depleted of mast cells was incubated with various concentrations of the mast cell mediators and rat IgG antibody-coated schistosomula (Fig. 2a). A highly significant increase in the levels of cytotoxicity was obtained with the ECF-A tetrapeptides Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu and with the analogue Val-Pro-Ser-Glu at the three highest concentrations. A smaller effect was detected with Val-Gly-Ser-Glu-methyl ester, and histamine showed no significant effect. Comparison of the EA rat rosette and the IgG-dependent cytotoxicity assay results as a function of the dose of the tetrapeptides or histamine revealed a highly significant correlation ($r = 0.72$, $P < 0.001$; Fig. 2b).

To determine whether the Fc receptor enhancement was restricted to rat eosinophils, similar experiments were performed with human eosinophils. When blood eosinophils from patients with parasite-unrelated hypereosinophilic syndromes were preincubated with various mediators, an increase in the percentage of rosettes was obtained both with EA rat and with EA rabbit, but the effect was significant only for Ala-Gly-Ser-Glu (Fig. 3a). When eosinophils were prepared from normal subjects, the increase in rosettes was very pronounced in the case of EA rat with Ala-Gly-Ser-Glu, Val-Gly-Ser-Glu and Val-Pro-Ser-Glu and was also significant with EA rabbit in the presence of Ala-Gly-Ser-Glu (Fig. 3b); however, neither Val-

Gly-Ser-Glu-methyl ester nor histamine had a significant effect. The specificity of the effect for eosinophil IgG Fc receptors was investigated by examining the formation of EA rat and EA rabbit rosettes by rat and human neutrophils (Table 1). In both cases, the percentage of rosettes was higher with EA rabbit than with EA rat and none of the ECF-A tetrapeptide analogues significantly increased the extent of rosette formation. In contrast, 10⁻⁴ M histamine significantly suppressed the formation of EA rat rosettes with rat neutrophils without affecting the formation of other neutrophil rosettes.

The data indicate that the human ECF-A tetrapeptides Ala⁴ and Val⁴, and the analogue Pro³ markedly increase the number of Fc receptors for rat IgG on the rat eosinophil membrane. The enhancement of the expression of IgG Fc receptors on eosinophils and the augmentation of the cytotoxic capacity of rat eosinophils for IgG-coated schistosomula exhibit similar dose-response relationships, suggesting that the two phenomena are related on a fundamental cellular level. The incubation of human eosinophils with Ala-Gly-Ser-Glu and, in some cases, with Val-Pro-Ser-Glu and Val-Gly-Ser-Glu, also increased the expression of Fc receptors, whereas Val-Gly-Ser-Glu-methyl ester and histamine were inactive. Previous reports have shown no enhancement of the expression of human eosinophil IgG receptors by the ECF-A tetrapeptides, whereas complement receptors were increased by both the ECF-A tetrapeptides and histamine^{8,9}. These earlier studies mainly used eosinophils from hypereosinophilic subjects and erythrocytes sensitized with rabbit antibodies. The present work clearly shows that when eosinophils from patients with hypereosinophilia were compared with normals for rosette formation with EA rabbit (Fig. 3), the patients had a higher proportion of eosinophils expressing Fc receptors, such that the increase by the tetrapeptides was only significant for one of the tetrapeptides studied. The major difference between the present report and previous studies limited to eosinophils from hypereosinophilic patients may therefore be explained by the fact that a significant increase could not be seen in the latter case because the starting percentage of Fc receptor expression was higher. It seems likely that the use of normal human eosinophils and EA-IgG rat unveiled the effects of the ECF-A tetrapeptides on the expression of eosinophil Fc receptors. Another possible explanation for the difference between the results of the two studies concerning EA-IgG rat and EA-IgG rabbit could be the preferential stimulatory role of ECF-A on surface receptors for anaphylactic antibodies which represent a more important part of rat IgG than rabbit IgG. This hypothesis is now being tested.

The large difference in the percentage of neutrophils that formed rosettes with EA rat compared with EA rabbit indicates that both the rat and human neutrophil Fc receptor seemed to possess a higher affinity for rabbit antibody than for rat anti-

Table 1 Effect of ECF-A tetrapeptides, analogues and histamine on neutrophil rosettes

Incubation with	% Rat neutrophil rosettes		% Human neutrophil rosettes	
	EA rat	EA rabbit	EA rat	EA rabbit
Medium	25.6 $\pm$ 3.2	94.0 $\pm$ 2.1	14.2 $\pm$ 6.5	99.6 $\pm$ 0.4
Ala-Gly-Ser-Glu	28.8 $\pm$ 5.4	93.3 $\pm$ 6.6	22.8 $\pm$ 3.9	100.0 $\pm$ 0
Val-Gly-Ser-Glu	34.0 $\pm$ 7.8	79.2 $\pm$ 3.9	12.5 $\pm$ 4.7	95.0 $\pm$ 5.0
Val-Pro-Ser-Glu	18.7 $\pm$ 5.3	90.2 $\pm$ 9.1	16.4 $\pm$ 6.2	98.8 $\pm$ 0.8
Val-Gly-Ser-Glu-methyl ester	23.7 $\pm$ 5.9	82.0 $\pm$ 9.4	15.2 $\pm$ 6.6	82.5 $\pm$ 12.7
Histamine	6.6 $\pm$ 1.7*	91.3 $\pm$ 3.7	10.3 $\pm$ 1.2	100.0 $\pm$ 0

Rat neutrophils were obtained from the peritoneal cavity of rats 7 h after the interperitoneal injection of 10 ml of 10% proteose-peptone in saline. Human neutrophils were prepared from venous blood from hypereosinophilic or normal subjects according to ref. 5. Rat or human neutrophils were preincubated for 60 min at 37 °C with the various mediators at a final concentration of 10⁻⁴ mol l⁻¹. The values given represent the mean of five experiments $\pm$ s.e.

*The extent of suppression was significant at $P < 0.01$.

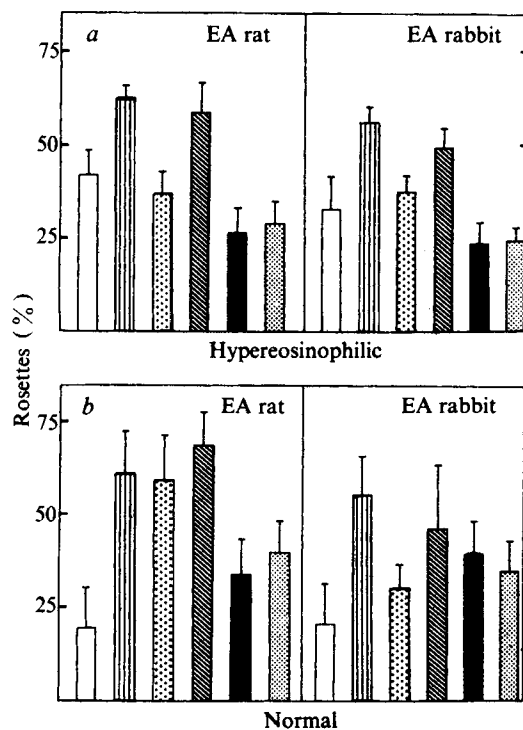


Fig. 3 The effect of the ECF-A tetrapeptides Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu, the analogues Val-Pro-Ser-Glu and Val-Gly-Ser-Glu-methyl ester, and histamine on EA rat and EA rabbit rosette formation by human eosinophils. Eosinophils were prepared from venous blood of nine patients with eosinophilia of non-parasitic aetiology (a) and from that of four normal subjects (b) by centrifugation on metrizamide discontinuous gradients⁸. 80–100% pure eosinophils were obtained from both groups of individuals. EA rat and EA rabbit were prepared according to Fig. 1. □, medium; ▨, Ala-Gly-Ser-Glu; ▤, Val-Gly-Ser-Glu; ▩, Val-Pro-Ser-Glu; ■, Val-Gly-Ser-Glu-methyl ester; ●, histamine. The results are expressed as the mean of triplicate experiments (±s.e.) and were compared by a Student's *t*-test. a, The increase in EA rat and EA rabbit rosettes was significant with Ala-Gly-Ser-Glu ($P < 0.05$). b, The increase in EA rat rosettes was significant with Ala-Gly-Ser-Glu, Val-Gly-Ser-Glu and Val-Pro-Ser-Glu ($P < 0.05$) whereas only Ala-Gly-Ser-Glu significantly increased EA rabbit rosettes ($P < 0.05$).

body. The mast cell mediators that increased the expression of eosinophil Fc receptors did not enhance either rat or human neutrophil Fc receptors, even when the percentage of Fc receptors expressed by normal neutrophils was very low (EA rat rosettes). These results therefore suggest that the effect of the tetrapeptides is specific for eosinophils.

The enhancement and inhibition of eosinophil IgG Fc receptors by mast cell-derived mediators may be a function of alterations in the rate of induction of new receptors or in the exposure of previously 'hidden' receptors, as has been suggested for complement receptors^{9,10}. It has been observed that the number of erythrocytes per eosinophil is also increased, when the percentage of rosetting eosinophils is enhanced by the tetrapeptides. Further, a correlation exists between the eosinophil chemotactic activity of the tetrapeptides and their effects on the expression of IgG Fc receptors on the eosinophil membrane¹¹. The parent tetrapeptides Ala⁴ and Val⁴ and the Pro³ analogue exhibit the highest activity in the three assays that have been examined, namely chemotaxis, rosette formation and cytotoxicity, whereas histamine is less active or inhibitory at comparable concentrations. A relationship between surface binding sites for chemotactic factors and for the Fc fraction of IgG has been envisioned for lymphocytes¹². Similarly, it is possible that the enhancement or inhibition of expression of eosinophil IgG Fc receptors by chemotactic factors involves an interaction between surface receptors common for chemotactic and IgG Fc binding functions, or with distinct receptors that are concurrently activated by the chemotactic factors. The capacity of the tetrapeptides of ECF-A to enhance the expression of eosinophil C3b (ref. 8) as well as IgG Fc receptors supports the latter possibility and suggests that modification of the properties

of receptors may be a general membrane response of eosinophils to some chemotactic factors. The ability of defined chemotactic factors to modulate both the migration of the eosinophils and their expression of surface receptors for immunologically relevant principles indicates a more general role for chemotactic factors that should be explored for other classes of leukocytes.

A recent study reports the increased capacity of eosinophils to bind to IgG-coated particles *in vitro* when exposed to various stimuli such as soluble immune complexes or endotoxin¹³. Results of this and the present study therefore suggest possible mechanisms to account for the higher proportion of eosinophils with Fc receptors in hypereosinophilic syndromes.

The present work is thus the first demonstration that mast cell mediators like the ECF-A tetrapeptides can not only promote eosinophil recruitment but also increase IgG-mediated eosinophil cytotoxicity against *Schistosoma* targets, by enhancing the expression of eosinophil IgG Fc receptors. These results, combined with previous studies on the enhancement of complement receptors and complement-mediated eosinophil cytotoxicity¹⁰, provide novel evidence for mast cell-eosinophil interaction in immunity against helminthic parasites.

We thank Professor M. Goudemand, Professor A. B. Tonnel and Dr L. Prin for providing human eosinophils, and J. P. Papin and S. Torres for technical assistance. This work was supported by INSERM U 167 and CNRS ERA 422.

Received 9 July; accepted 4 November 1980.

- Goetzl, E. J. & Austen, K. F. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4123–4127 (1975).
- Clark, R. A. F., Gallin, J. I. & Kaplan, A. P. *J. exp. Med.* **142**, 1462–1476 (1975).
- Smith, J. A., Goetzl, E. J. & Austen, K. F. in *Peptides: Structure and Biological Function* (eds Meienhofer, J. & Gross, E.) (Pierce Chemical Co., Rockford, Illinois, in the press).
- Capron, M., Rousseaues, J., Mazingue, C., Bazin, H. & Capron, A. *J. Immun.* **121**, 2518–2525 (1978).
- Vadas, M., David, J. R., Butterworth, A. E., Pisani, N. T. & Siongok, T. A. *J. Immun.* **122**, 1228–1236 (1979).
- Capron, M., Torpier, G. & Capron, A. *J. Immun.* **123**, 2220–2230 (1979).
- Butterworth, A. E., Coombs, R. R. A., Gurner, B. W. & Wilson, A. B. *Int. Archs Allergy appl. Immun.* **51**, 368–377 (1976).
- Anwar, A. R. E. & Kay, A. B. *Nature* **269**, 522–524 (1977).
- Anwar, A. R. E. & Kay, A. B. *J. Immun.* **121**, 1245–1250 (1978).
- Kay, A. B. *J. Allergy clin. Immun.* **64**, 90–104 (1979).
- Capron, M., Goetzl, E. J., Austen, K. F. & Capron, A. *Prog. Immun.* (in the press).
- Shields, J. M. & Wilkinson, P. C. *Clin. exp. Immun.* **38**, 598–608 (1979).
- Tai, P. C. & Spry, C. J. F. *Clin. exp. Immun.* **40**, 206–219 (1980).
- Lynch, S. M., Austen, K. F. & Wasserman, S. I. *J. Immun.* **121**, 1394–1399 (1978).

Synaptic localization of kainic acid binding sites

A. C. Foster, E. E. Mena, D. T. Monaghan & C. W. Cotman

Department of Psychobiology, University of California, Irvine, Irvine, California 92717

The heterocyclic compound kainic acid (KA) is a potent excitant when applied to mammalian neurones¹. Lesions caused by injections of KA into the rat striatum and hippocampus cause similar patterns of damage to those seen in Huntington's chorea and status epilepticus, respectively^{2,3}. Although it was originally thought to be a glutamate agonist⁴, it is now clear that KA does not act on the majority of the receptors for glutamate^{5–7}, and in fact seems to act on a class of receptors which are distinct from those which mediate responses to other excitatory amino acids^{8,9}. The potent and selective neurotoxic effects of this compound¹⁰ may be mediated by these same receptors. At present, the relative distribution of junctional and extra-junctional (non-synaptic) receptors is unknown and resolution of this issue would provide important insights into the action of KA on the central nervous system (CNS). We show here that KA binding sites are greatly enriched in isolated synaptic junctions from rat brain and, using an *in vitro* autoradiographic technique, we have found that these binding sites are concentrated specifically in terminal fields where KA acts as a potent neurotoxin.

Synaptic junctions (SJs) and other subcellular fractions were prepared by the method of Cotman and Taylor¹¹, and assayed for ³H-KA binding^{12,13}. Table 1 shows the relative values for specific binding (the ³H-KA binding which can be displaced by 100 μ M unlabelled KA) in the isolated fractions, which are expressed as a value relative to the whole particulate fraction (=1). Membranes from crude mitochondrial pellet (P₂) were not enriched in KA binding sites relative to the whole particulate fraction. However, on subfractionation of P₂, the highest specific binding values were found in the synaptic plasma membranes (SPMs), in agreement with previous results¹³. The P₁, P₃, myelin enriched and mitochondria enriched fractions also had specific KA binding sites. This may be due to the presence of synaptic plasma membranes in these fractions, and indeed both purified myelin and mitochondria have been found to contain lower levels of high-affinity binding sites for ³H-KA¹³.

Specific KA binding sites in SJs were enriched 22 times over those measured in the whole brain particulate fraction, and 10 times over those in SPMs. This enrichment in SJ fractions is greater than that shown previously for any other receptor or receptor-related function. The absolute amount of binding to SJs, averaged over four separate preparations and assayed at a concentration of 40 nM, was $1,819 \pm 378$ fmol per mg protein. The majority of specific KA binding sites in the SPMs could be recovered in SJs ($73 \pm 14\%$ of binding sites; $9.4 \pm 0.6\%$ protein; $n = 4$); which suggests that most of the binding sites for KA in SPMs are located at the junctional area. Similarly, 80% of the binding sites in SPMs for L-glutamate and L-aspartate are recovered in SJs; quinuclidinyl benzoate binding sites are not detectable in SJs prepared by this method.

Two components of KA binding were apparent in SJs (assayed over a ³H-KA concentration range of 1–100 nM). The mean values from two determinations measured for the high-affinity component were $K_D = 4.95$ nM and $B_{max} = 1,349$ fmol per mg protein, and for the low-affinity component 32.6 nM and 3,257 fmol per mg protein, respectively. These values for the K_D s agree with those previously reported in crude membrane preparations¹⁴, and indicate that the increase of KA binding seen in SJs is a true enrichment of sites and is not due to an increase in affinity caused, for example, by removal of an endogenous inhibitor.

If KA binding sites are predominantly synaptic, they might be localized to discrete terminal fields. KA binding sites can be viewed directly in rat brain slices by an *in vitro* autoradiographic technique¹⁵. Using this method, KA binding sites were seen to be particularly enriched in discrete parts of the hippocampus, pyriform cortex, cerebellum and striatum. In the hippocampus a small band of silver grains was associated with the inner part of the molecular layer of the dentate gyrus, corresponding to the terminal field of the commissural/associational fibres. However, the greatest density of grains was seen over the stratum incidium in the CA3 region corresponding to the terminal zone of the

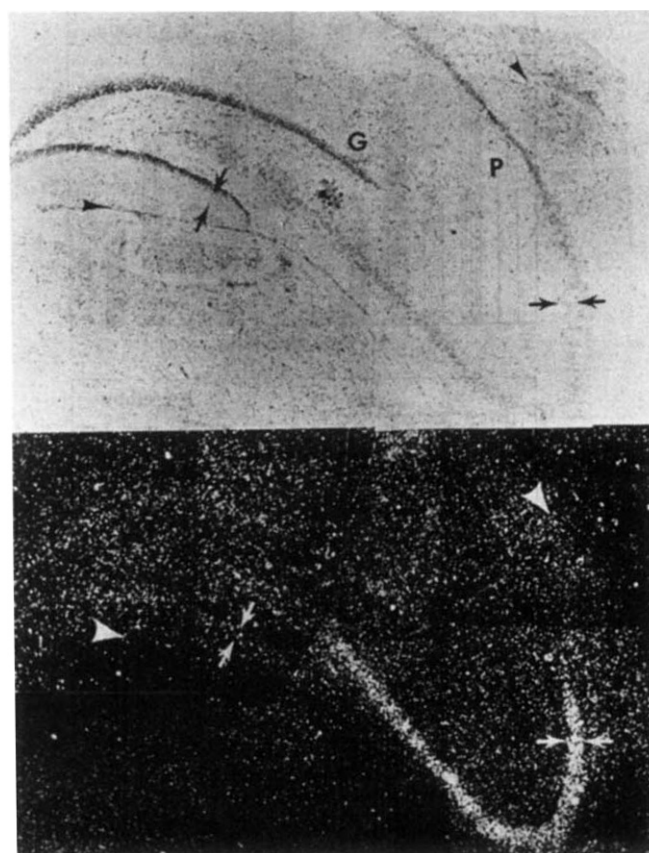


Fig. 1 Distribution of KA binding sites in the rat hippocampus. Brain slices (15 μ m) from adult Sprague-Dawley rats were sliced on a cryostat, mounted unfixed on glass microscope slides and processed for the localization of binding sites using a modification of the method of Young and Kuhar¹⁵. ³H-KA was used at a final concentration of 100 nM, and incubated at 0°C for 30 min. Bottom: dark field illumination, small arrows indicate the areas of high silver grain density. Top: cresyl violet-stained tissue section; small arrows indicate identical area in dark field possessing high silver grain density. G, granule cells; P, pyramidal cells.

mossy fibre input to the CA3 pyramidal cells (Fig. 1). The KA binding sites labelled by this technique were apparently identical to those revealed in membrane binding assays, as this pattern of binding could be completely displaced with an excess of unlabelled KA, and binding to these slices was saturable with a K_D value in the range found for SJs. The pattern of KA binding sites we have observed in the hippocampus is particularly interesting in view of the previous reports that the CA3 pyramidal cells of the hippocampus are extremely sensitive to the neurotoxic action of KA¹⁶, particularly at the mossy fibre terminal zone¹⁷. Other brain areas also showed KA sites but in general these were of lower concentration than the CA3 field (manuscript in preparation).

The results presented here, therefore, suggest a discrete localization of KA binding sites in the mammalian CNS. At a subcellular level, SJs contribute the majority of the 'synaptic population' of KA binding sites, and in the hippocampus these sites are present in high density in close apposition to the mossy fibre-CA3 pyramidal cell synapse. Neither glutamate nor aspartate seem to be transmitters at this synapse¹⁸. Specific and localized synaptic receptors imply that appropriate natural ligands exist. Our data, together with evidence that KA does not act on glutamate sites and that KA receptors are a discrete population^{8,9}, suggest that endogenous ligands for KA receptors exist. Such a compound would be a mediator or modulator of synaptic transmission at sites where we have shown KA receptors to exist. Furthermore, the identification of such compounds may provide new insights into the aetiology of Huntington's disease and epilepsy.

Table 1 Specific binding of ³H-KA to subcellular fractions of rat brain

Fraction	Specific ³ H-KA binding relative to OH (=1)	n
Whole particulate (OH)	1	4
Crude mitochondrial pellet (P ₂)	0.73 ± 0.34	4
Microsomes (P ₃)	0.56, 0.25	2
Crude myelin	0.05, 0.13	2
Synaptic plasma membrane (SPM)	2.08 ± 0.41	4
Mitochondria	0.95 ± 0.21	3
Synaptic junction (SJ)	22.37 ± 5.79	4

Subcellular fractions were prepared from whole rat forebrain as described by Cotman and Taylor¹¹. All fractions were washed in 0.2 mM HEPES, 0.05 mM CaCl₂ buffer (pH 7.4) and pelleted at 80,000g for 20 min. Binding assays were done in triplicate essentially as previously described¹³. Crude myelin was taken as the fraction floating on the 0.8 M sucrose layer. The mitochondria fraction was the 1.2 M pellet.

This work was supported by NIH and NIMH grants. A.C.F. holds a NATO postdoctoral fellowship and E.E.M. a NIH postdoctoral fellowship. We thank Mrs D. Franks for assistance.

Received 2 September; accepted 27 October 1980.

- Shinozaki, H. & Konishi, S. *Brain Res.* **24**, 368–371 (1970).
- Coyle, J. T., McGeer, E. G., McGeer, P. L. & Schwarcz, R. in *Kainic Acid as a Tool in Neurobiology* (eds McGeer, E. G., Olney, J. W. & McGeer, P. L.) 139–160 (Raven, New York, 1978).
- Nadler, J. V., Perry, B. W. & Cotman, C. W. in *Kainic Acid as a Tool in Neurobiology* (eds McGeer, E. G., Olney, J. W. & McGeer, P. L.) 219–238 (Raven, New York, 1978).
- Johnston, G. A. R., Curtis, D. R., Davies, J. & McCulloch, R. M. *Nature* **248**, 804–805 (1974).
- Hall, J. G., Hicks, T. P. & McLennan, H. *Neurosci. Lett.* **8**, 171–175 (1978).
- Foster, A. C. & Roberts, P. J. *Neurochemistry* **31**, 1467–1477 (1978).
- London, E. D. & Coyle, J. T. *Eur. J. Pharmac.* **56**, 287–290 (1979).
- Davies, J., Evans, R. H., Francis, A. A., Jones, A. W. & Watkins, J. C. in *EMBO Workshop on Drug Receptors in the Central Nervous System* (ed. Balaban, M.) (Int. Sci. Services, Jerusalem, 1980).
- McLennan, H. & Lodge, D. *Brain Res.* **169**, 83–90 (1979).
- McGeer, E. G., Olney, J. W. & McGeer, P. L. (eds) *Kainic Acid as a Tool in Neurobiology* (Raven, New York, 1978).
- Cotman, C. W. & Taylor, D. J. *Cell Biol.* **55**, 696–711 (1972).
- Simon, J. R., Contrera, J. F. & Kuhar, M. J. *J. Neurochem.* **26**, 141–147 (1976).
- Nieto-Sampedro, M., Shelton, D. & Cotman, C. W. *Neurochem. Res.* **5**, 591–604 (1980).
- London, E. D. & Coyle, J. T. *Molec. Pharmac.* **15**, 492–505 (1979).
- Young, W. S. & Kuhar, M. J. *Brain Res.* **179**, 255–270 (1979).
- Nadler, J. V., White, W. F., Vaca, K. W. & Cotman, C. W. *Nature* **271**, 676–677 (1978).
- Olney, J. W., Fuller, T. & DeGubareff, T. *Brain Res.* **176**, 91–100 (1979).
- Nadler, J. V., White, W. F., Vaca, K. W., Perry, B. W. & Cotman, C. W. *J. Neurochem.* **31**, 147–155 (1978).

Corticotropin regulates trans-synaptically the activity of septo-hippocampal cholinergic neurones

Lawrence J. Botticelli* & Richard J. Wurtman

Laboratory of Neuroendocrine Regulation, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

The activity of septo-hippocampal neurones is affected by the action on cholinergic perikarya in the septum of a variety of putative neurotransmitters^{1–4}, including substance P^{5,6} and β -endorphin^{7,8}. (The latter is released in the septal region from neurones which originate in the medial basal hypothalamus.) It has also been reported that two other neuropeptides, corticotropin (ACTH_{1–24}) and α -melanotropin (α -MSH), affect acetylcholine turnover in septo-hippocampal neurones in a manner that is not blocked by transection of the afferents to the hippocampus, from which it has been inferred that the neurotransmitters act directly on the hippocampus⁹. We now describe experiments with corticotropin which show that the effect is rather the influence on septo-hippocampal cholinergic neurones of peptidergic neurones within the septum.

Male Sprague-Dawley rats (150–200 g, Charles River Breeding Laboratories) were housed in groups of three to five at an ambient temperature of 23–24°C for 5–7 days before experimentation. Animals had free access to food (Purina Rat chow; Ralston-Purina) and water, and were exposed to light (Vita-Lite, 300 μ W cm⁻²; Duro Test Corporation) daily between 0700 and 1900 h.

A polyethylene tube was implanted in the lateral ventricle (2 mm lateral, 1 mm posterior to bregma and 3 mm ventral to the dural surface) of each rat 3 days before experimentation. Cannulae were kept in position with dental cement. For intraseptal injections, guide cannulae were implanted stereotactically into the septum (coordinates AP 8.5, ML 0.0, DV 0.0, 5.0 mm ventral to the dural surface) 5 days before experimentation. For intrahippocampal injections, cannulae were placed in the dorsal hippocampus (coordinates AP 4.0, ML 2.0, DV 2.0) 3 days before experimentation. Cannulae placements were verified histologically by staining a 1:3 or 1:5 series of

Table 1 Effects of peptides on hippocampal acetylcholine (ACh) content

Treatment	Hippocampal ACh (pmol per mg)
Vehicle	40.0 $\pm$ 2.3 (n = 6)
β -lipotropin	37.5 $\pm$ 1.7 (n = 5)
β -endorphin	51.4 $\pm$ 2.7* (n = 5)
Corticotropin	25.5 $\pm$ 1.5* (n = 5)
ACTH _{4–10}	38.7 $\pm$ 2.9 (n = 5)
α -MSH	39.9 $\pm$ 2.2 (n = 5)

Rats were killed 30 min after intraventricular administration of each peptide. Values are expressed as means $\pm$ s.e.m. Data were analysed by analysis of variance (corrected for unequal sample size), followed by *a posteriori* multiple comparisons of within-group means using Scheffe's method¹¹.

* $P < 0.001$ differs from vehicle-treated animals (*a posteriori*).

40- μ m frozen frontal cryostat sections with thionin. For peptide co-administration studies, rats were equipped with both intraventricular and intraseptal cannulae.

Peptides dissolved in pyrogen-free water were microinjected into the lateral ventricle (10 μ g, into the septum (1.0 μ g in 1.0 μ l given over 5 min), or the dorsal hippocampus (1.0 μ g in 1.0 μ l given over 3 min). At 30 min after peptide micro-administration the animals were killed by a beam of microwave irradiation directed at the skull. After decapitation, brains were removed from the skull and specimens of cerebral cortex, striatum and hippocampus (dorsal) dissected by hand; their acetylcholine and choline content was determined radioenzymatically using [γ -³²P]ATP (specific activity 10–30 Ci nmol⁻¹; Amersham) as labelled substrate¹⁰. Significance of treatment-induced differences in regional brain acetylcholine or choline content was determined by analysis of variance (Wang 2200 series software; Wang Laboratories), followed by *a posteriori* multiple comparisons of within-group means using Scheffe's method¹¹.

Acetylcholine content in cortex, striatum and hippocampus was unchanged after intraventricular administration of β -LPH (Table 1). As described previously⁸, intraventricular β -endorphin significantly increased acetylcholine content in the hippocampus (38%; $P < 0.001$) (Table 1) but not elsewhere. Corticotropin had an opposite effect on hippocampal acetylcholine concentrations, causing them to fall by about 30% ($P < 0.001$) (Table 1), but like β -endorphin, failed to affect acetylcholine levels in the two other brain regions examined. Neither ACTH_{4–10} nor α -MSH affected acetylcholine concentrations in any brain region examined (Table 1). None of the peptides affected choline levels in any region (data not shown).

When smaller doses (1 μ g) of β -endorphin or corticotropin were microinjected directly into the septum, hippocampal acetylcholine levels changed in the same direction as after its

Table 2 Effects of β -endorphin and/or corticotropin on hippocampal acetylcholine

Treatment	Hippocampal ACh (pmol per mg)
Vehicle	37.7 $\pm$ 1.2 (n = 4)
β -endorphin (i.s.t.)	58.4 $\pm$ 3.0* (n = 5)
Corticotropin (i.s.t.)	21.6 $\pm$ 2.4* (n = 5)
β -endorphin + corticotropin (i.s.t.)	42.0 $\pm$ 3.1† (n = 6)
β -endorphin (i.v.t.) + corticotropin (i.s.t.)	39.9 $\pm$ 1.3† (n = 6)
β -endorphin (i.s.t.) + corticotropin (i.v.t.)	37.9 $\pm$ 2.6† (n = 6)

Rats were killed 30 min after injection of β -endorphin and corticotropin [either both intraseptally (i.s.t.) (0.5 μ g each) or one intraseptally (1.0 μ g) and the other intraventricularly (i.v.t.) (10 μ g)]. Values are expressed as means $\pm$ s.e.m. Data were analysed by analysis of variance (corrected for unequal sample size) followed by *a posteriori* multiple comparisons of within-group means using Scheffe's method¹¹.

* $P < 0.001$ differs from vehicle-treated animals (*a posteriori*).

† $P < 0.001$ differs from peptide-treated animals (*a posteriori*).

* Present address: Addiction Research Foundation, 701 Welch Road, Palo Alto, California 94304.

intraventricular injection but to a greater extent: β -endorphin administration increased acetylcholine concentrations by 56% ($P < 0.001$), whereas corticotropin decreased hippocampal acetylcholine by 44% ($P < 0.001$) (Table 2). Co-administration of the neuropeptides, either both intraseptally (0.5 μ g each), or one intraseptally (1.0 μ g) and the other intraventricularly (10 μ g), did not significantly change hippocampal acetylcholine levels (Table 2). Direct administration of β -endorphin or corticotropin into the dorsal hippocampus (1.0 μ g) also failed to affect hippocampal acetylcholine (Table 3).

Corticotropin and β -endorphin, which may share a common precursor¹², have similar brain distributions; they are concentrated within a major cell group in the periaruate hypothalamus, projecting to the anterior hypothalamus, pons, the periaqueductal grey and the septal region¹³⁻¹⁵. Nerve fibres immunoreactive for both corticotropin and β -endorphin surround the third ventricle, extend throughout the diencephalon and portions of the limbic brain, and descend into the central grey. No corticotropin or endorphin immunoreactivity has been detected in the hippocampus, cerebrum or cerebellar cortices. Corticotropin and β -endorphin can sometimes block each

Table 3 Effects of intrahippocampal β -endorphin or corticotropin on hippocampal acetylcholine

Treatment	Hippocampal ACh (pmol per mg)
Vehicle	39.6 $\pm$ 2.1 ($n = 5$)
β -endorphin	43.6 $\pm$ 1.5 ($n = 5$)
Corticotropin	41.9 $\pm$ 2.8 ($n = 5$)

Rats were killed 30 min after injection of peptides into the dorsal hippocampus (1.0 μ g per 1.0 μ l for 3.0 min). Values are expressed as means $\pm$ s.e.m.

other's effects, or produce effects opposite to one another¹⁶⁻¹⁸, which suggests that these peptidergic neurones may participate reciprocally in common neuronal systems.

The activity of septo-hippocampal neurones is controlled partly by axosomatic and axodendritic synapses in the septum itself, and partly by hippocampal axoaxonic synapses. Intraseptally injected β -endorphin produces a naloxone-reversible decrease in the turnover of hippocampal acetylcholine⁷, suggesting that endorphin interacts with opiate receptors in the septal region. In contrast, corticotropin has been reported to increase the turnover of this neurotransmitter in Ammon's horn, even after transection of the cingulum, entorhinal area and fimbria⁹. Such observations have been interpreted as suggesting a direct action of these neuropeptides on receptors in the hippocampal formation. Our observations that intraseptal corticotropin decreases hippocampal acetylcholine, that intrahippocampal corticotropin fails to change the content of hippocampal acetylcholine and that co-administered β -endorphin and corticotropin block each other's effects on hippocampal acetylcholine, imply that corticotropin-containing peptidergic neurones do act within the septum, and thereby influence the physiological activity of septo-hippocampal cholinergic neurones.

These studies were supported in part by research grants awarded to R.J.W. from the National Institute of Mental Health (MH 28783), the Ford Foundation and the Wallace Genetic Foundation. L.J.B. was a predoctoral fellow of the National Institute on Drug Abuse (DA 05089). β -Lipotropin was supplied by Dr C. H. Li, β -endorphin by the Psychopharmacology Research Branch, National Institute of Mental Health, corticotropin, ACTH₄₋₁₀ and α -MSH were gifts of Organon Pharmaceuticals, and all other chemicals used were purchased from either Eastman Organic or Aldrich.

Received 15 September; accepted 24 October 1980.

- McLennan, H. & Miller, J. J. *J. Physiol., Lond.* **237**, 607-624, 624-633 (1974).
- DeFrance, J. F., Yoshihara, H., McCrea, R. A. & Kitai, S. T. *Expl Neurol.* **48**, 502-523 (1975).
- Assaf, S. Y. & Miller, J. J. *Brain Res.* **129**, 353-360 (1977).

- Moore, R. Y. *J. comp. Neurol.* **177**, 665-684 (1978).
- Cuello, A. C. & Kanazawa, I. *J. comp. Neurol.* **178**, 129-156 (1978).
- Malthe-Sorensen, D., Cheney, D. L. & Costa, E. *J. Pharmac. exp. Ther.* **206**, 21-28 (1978).
- Moroni, F., Cheney, D. L. & Costa, E. *Naunyn-Schmiedeberg Archs Pharmac.* **299**, 149-153 (1977).
- Botticelli, L. J. & Wurtman, R. J. *Life Sci.* **24**, 1799-1804 (1979).
- Wood, P. L., Cheney, D. L. & Costa, E. *J. Pharmac. exp. Ther.* **209**, 97-103 (1979).
- Goldberg, A. M. & McCaman, R. E. *J. Neurochem.* **20**, 1-8 (1973).
- Winer, B. *Statistical Principles in Experimental Design*, 205-210 (McGraw-Hill, New York, 1971).
- Eipper, B. A. & Mains, R. E. *Endocr. Rev.* **1**, 1-27 (1980).
- Bloom, F. E. *et al.* in *The Endorphins* (eds Costa, E. & Trabucchi, M.) 89-109 (Raven, New York, 1980).
- Watson, S. J., Akil, H., Richards, C. W. III & Barchas, J. D. *Nature* **275**, 226-228 (1978).
- Watson, S. J., Richards, C. W. III & Barchas, J. D. *Science* **200**, 1180-1182 (1978).
- Jacquet, Y. F. *Science* **201**, 1032-1034 (1978).
- Simantov, R. *Nature* **280**, 684-685 (1979).
- Botticelli, L. J. & Wurtman, R. J. in *Endogenous and Exogenous Opiate Agonists and Antagonists* (ed. Leong Way, E.) 187-190 (Pergamon, New York, 1980).

Serotonin stimulates phosphorylation of Protein I in the facial motor nucleus of rat brain

Annette C. Dolphin* & Paul Greengard†

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

Protein I is one of the best candidates for a neuronal protein whose phosphorylation may have a functional role in synaptic activity¹. It is a substrate for both cyclic AMP-dependent² and Ca²⁺-dependent³ protein kinases, and these kinases show differential specificity for its multiple phosphorylation sites⁴. Protein I is found exclusively in the central⁵ and peripheral⁶ nervous systems, and immunohistochemical⁵ and subcellular fractionation⁷ studies suggest an association primarily with synaptic vesicles. Using slices of rat cerebral cortex incubated *in vitro*, Protein I was phosphorylated both by agents which increase intracellular cyclic AMP and by agents causing Ca²⁺ influx, although not by any putative neurotransmitters or neuromodulators⁸. We have now examined the facial motor nucleus and report here that serotonin produces a phosphorylation of Protein I when incubated with facial nucleus slices. Demonstration of a neurotransmitter-dependent alteration in the state of phosphorylation of a synapse-specific protein may be due to the relatively simple neuronal circuitry within the facial motor nucleus.

The facial motor nucleus contains a single class of neuronal cell bodies and no interneurons^{9,10}. Most of the afferent fibres are excitatory onto the facial motoneurons¹⁰ and stimulation of motoneuron firing is mimicked by the iontophoresis of glutamate¹⁰. The facial nucleus also receives a facilitatory serotonergic input from the raphe magnus¹⁰⁻¹², which represents about 2% of the terminals in the nucleus¹³.

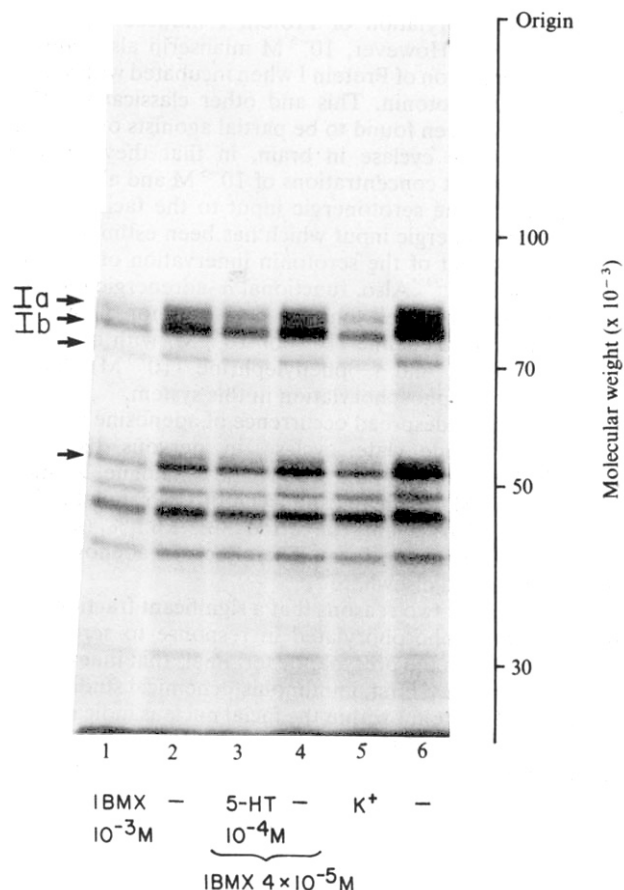
Slices prepared from excised rat facial nuclei were preincubated for 1 h in Krebs-Ringer bicarbonate buffer (KRB). The slices were then incubated for 1-20 min in the presence of serotonin or other agents. The reaction was terminated by homogenizing the tissue in zinc acetate (5 mM), which inhibits phosphatase activity⁸. Protein I was quantitatively extracted and subjected to 'back-phosphorylation' by the catalytic subunit of cyclic AMP-dependent protein kinase, using [γ -³²P]ATP as substrate. This method of back-phosphorylation gives a measure of the amount of dephospho-Protein I remaining in the slice at the end of the incubation period, from which one can calculate the amount of Protein I that was converted to the phosphorylated form during incubation with the various agents⁸ (see Figs 1 and 2 legends).

In resting conditions, after preincubation of the slices for 1 h, Protein I seemed to be almost entirely in the dephosphorylated

* Present address: Division of Neurophysiology and Neuropharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

† To whom reprint requests should be addressed.

Fig. 1 Autoradiogram of an SDS-polyacrylamide gel showing the effect of IBMX, of serotonin (5-HT) and of high K^+ on protein phosphorylation in slices of rat facial nucleus. Male Sprague-Dawley rats (150–250 g) were killed by decapitation. The brain was removed and a transverse slice, 1.2 mm thick, was taken through the brain stem immediately posterior to the trapezoid body. The regions containing the facial nuclei were then dissected manually as cubes taken from the ventral edge of the slice with the pyramidal tracts as the medial borders. Each excised nucleus was quartered using a sagittal and a horizontal cut. The four slices from a single facial nucleus were transferred together to a glass homogenizer containing 10 ml of KRB, pH 7.4, at 35 °C (composition in mM: Na^+ , 158; K^+ , 3; Ca^{2+} , 1.5; Mg^{2+} , 1.3; PO_4^{3-} , 1.3; HCO_3^- , 26; Cl^- , 136; ascorbate, 1; D-glucose, 20). The medium was continuously gassed with H_2O -saturated 95% O_2 -5% CO_2 throughout the preincubation and incubation periods. Slices were preincubated for 1 h with one change of medium. In all cases, the effect of the agent to be tested was determined by incubating it for 1 or 10 min with one of a pair of facial nuclei, the other of which was used as a control. Substances to be tested were added in KRB such that the final incubation volume was 10 ml. The incubations were terminated by aspirating the KRB, adding 5 ml of ice-cold Zn-acetate (5 mM) and homogenizing with a Teflon pestle. The amount of Protein I present in the dephospho-form at the termination of the incubation was determined by back-phosphorylation as follows: the homogenate was centrifuged at 4,000g for 15 min and the pellet resuspended in 1 ml of 10 mM Na-citrate-phosphate buffer, pH 2.8, at 4 °C. Triplicate 100- μ l aliquots of this suspension were taken for protein assay using a method with which monoamines did not interfere³¹. The suspension was centrifuged at 23,000g for 15 min and 500 μ l of the supernatant was adjusted to pH 6 with 0.5 M Na_2HPO_4 and centrifuged at 15,000g for 15 min. All pH 6 supernatants from one experiment were then diluted to equal protein concentration with 10 mM Na-citrate-phosphate buffer (pH 6). Duplicate or triplicate 60- μ l aliquots were used in the back-phosphorylation assay containing (in 100- μ l final volume): 50 mM HEPES (pH 7.4), 10 mM Mg^{2+} , 1 mM EGTA, 1 mM EDTA, 3 μ M [γ - ^{32}P]ATP (specific activity 50–100 c.p.m. $fmol^{-1}$) and a low concentration (7.5 nM) of the purified catalytic subunit (C) of cyclic AMP-dependent protein kinase, prepared according to Beavo *et al.*³². The back-phosphorylation assay was carried out for 30 min at 30 °C and samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography as previously described⁹. In control experiments, it was established that the back-phosphorylation assay conditions used resulted in a maximal incorporation of ^{32}P into phospho-peptide 1 of Protein I, with negligible phosphorylation of other phosphorylation sites on Protein I (ref. 33, and A.C.D., unpublished observations). Phospho-peptide 1 is the only tryptic phosphopeptide derived from Protein I which shows a high affinity for phosphorylation by cyclic AMP-dependent protein kinase, although it is also phosphorylated in a Ca^{2+} -dependent manner³³. Protein I is indicated by two arrows. It consists of two subspecies Ia and Ib of M_r 86,000 and 80,000. Lanes 1 and 2 incubations were with or without 10^{-3} M IBMX for 10 min; lanes 3 and 4 incubations were with or without 10^{-4} M serotonin for 10 min, in the presence of a low concentration of IBMX (4×10^{-5} M) added to both incubation tubes 2 min before the start of incubation; lanes 5 and 6 incubations were in the presence or absence of 60 mM K^+ (K^+ being substituted isotonically for Na^+) for 1 min.



form. A 10-min incubation with serotonin (10^{-4} M) increased the phosphorylation of Protein I in facial nucleus slices (Fig. 1, lane 3 compared with 4), as did the phosphodiesterase (PDE) inhibitor isobutyl methylxanthine (IBMX) (10^{-3} M; Fig. 1, lane 1 compared with 2). Each of these agents raises cyclic AMP levels in facial nucleus slices¹⁴. Several other proteins also showed increases in their state of phosphorylation in response to incubation of slices with serotonin and IBMX, indicating that they are also endogenous substrates for cyclic AMP-dependent protein kinase. The latter demonstrations are reasonable in view of the methodology used for the back-phosphorylation assay, in which the low concentration of catalytic subunit used selectively phosphorylated only those substrate proteins with a high affinity for this enzyme. As neurotransmitters thought to act through cyclic AMP can cause numerous biological responses in their target cells (for example, alterations in membrane excitability, in intermediary metabolism and in protein synthesis), many different phosphoproteins are probably involved in these responses^{15,16}. Indeed, several endogenous substrates for cyclic AMP-dependent protein kinase have already been detected in isolated neuronal membrane preparations^{2,17–20}.

Incubation of facial nucleus slices with a high concentration of K^+ (60 mM) also increased the phosphorylation of Protein I and several other phosphoproteins (Fig. 1, lane 5 compared with 6). That high K^+ altered the state of phosphorylation of several of the substrate proteins affected by serotonin suggests that regulation of phosphorylation sites on proteins by both cyclic AMP- and Ca^{2+} -dependent protein kinases may not be unusual. In this regard, note that two of the affected proteins, indicated by arrows in Fig. 1, seem to be the same as the 74,000 and 55,000 molecular weight (M_r) proteins whose phosphorylation was affected by 8-bromo-cyclic AMP, high K^+ and veratridine in slices of rat cerebral cortex⁸.

The formation of phospho-Protein I from dephospho-Protein I, as a function of serotonin concentration, is shown in Fig. 2. The stimulation of phosphorylation caused by serotonin was potentiated by IBMX (Fig. 2) at a concentration (4×10^{-5} M) which alone caused little alteration in the state of Protein I phosphorylation. In the presence of this concentration of IBMX, the maximal effect of a 10-min incubation with serotonin (10^{-3} M) was to convert $30.1 \pm 3.7\%$ ($n = 9$) of Protein I from the dephospho- to the phospho-form, and the EC_{50} of serotonin was 4×10^{-6} M. The time course of Protein I phosphorylation by serotonin (10^{-3} M) in the presence of IBMX (4×10^{-5} M) was investigated and found to be similar to that observed for 10^{-3} M IBMX in cortical slices⁸. The conversion of dephospho-Protein I to phospho-Protein I was half-maximal after about 2 min, maximal after 5 min and remained constant for at least another 15 min.

That the maximal phosphorylation of Protein I in response to serotonin was not greater than 30% may be because, to excise the facial nucleus intact, it was necessary to take some tissue from adjacent regions which are not innervated by serotonergic terminals. Moreover, in all tissue-slice systems studied so far (for review, see ref. 15), 30–50% of Protein I has been observed to be refractory to phosphorylation in steady-state conditions by 8-bromo-cyclic AMP, IBMX, K^+ or veratridine, either alone or in combination. In the present study, incubation with 10^{-3} M IBMX for 10 min produced a $50.2 \pm 3.4\%$ ($n = 8$) phosphorylation of Protein I, and incubation of facial nucleus slices for 1 min with 60 mM K^+ -KRB produced a $48.5 \pm 3.6\%$ ($n = 7$) phosphorylation of Protein I. The nature and subcellular localization of the refractory Protein I is being investigated.

That serotonin affected Protein I phosphorylation by interaction with specific receptors was indicated by the ability of mianserin, an antagonist at central serotonin receptors^{21–23}, to

block the phosphorylation of Protein I induced by 10^{-4} M serotonin (Fig. 3). However, 10^{-5} M mianserin also caused a slight phosphorylation of Protein I when incubated with slices in the absence of serotonin. This and other classical serotonin antagonists have been found to be partial agonists of serotonin sensitive adenylate cyclase in brain, in that they stimulate adenylate cyclase at concentrations of 10^{-5} M and above²³.

In addition to the serotonergic input to the facial nucleus, there is a noradrenergic input which has been estimated to be about one-fifth that of the serotonin innervation of the facial nucleus in the rat^{10,24}. Also, functional α -adrenergic receptors have been demonstrated on the facial motor neurones¹⁰. However, (-)noradrenaline (10^{-3} or 10^{-4} M) with or without 4×10^{-5} M IBMX, and (-)phenylephrine (10^{-3} M), had no effect on Protein I phosphorylation in this system.

In view of the widespread occurrence of adenosine receptors associated with adenylate cyclase in nervous tissue, we examined the effect of the adenosine analogue 2-chloro-adenosine (10^{-3} M) on the phosphorylation of Protein I in facial nucleus slices. This agent converted $33.3 \pm 6.5\%$ ($n=4$) of Protein I to the phospho-form. The effects of adenosine analogues are reported elsewhere²⁵.

We conclude for two reasons that a significant fraction of the Protein I that is phosphorylated in response to serotonin is probably located in the presynaptic terminals that innervate the facial motoneurons. First, immunohistochemical studies of the distribution of Protein I within the facial nucleus indicate that it is present in most and possibly all nerve terminals, whereas it was not detectable in the motoneurone somata (P. De Camilli and A. C. D., unpublished results). Second, serotonin has been found to stimulate selectively the phosphorylation of phospho-

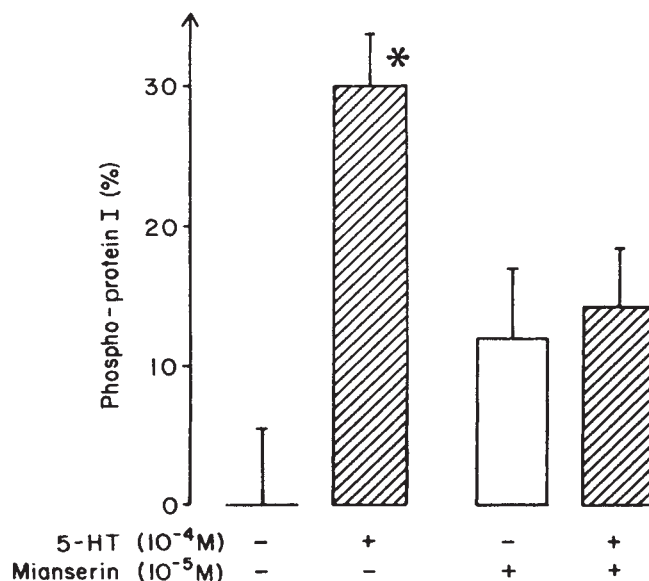


Fig. 3 The effect of the serotonin antagonist mianserin on the phosphorylation of Protein I in rat facial nucleus slices. One of each pair of facial nucleus slices was incubated with, and one without, serotonin (5-HT, 10^{-4} M) for 10 min. Mianserin (10^{-5} M), where included, was added to each pair 15 min before the end of the incubation. IBMX (4×10^{-5} M) was present in all incubation tubes and was added 12 min before the end of the incubation. The amount of dephospho- and phospho-Protein I was determined as described in Fig. 2 legend and was calculated with reference to slices incubated in the absence of both serotonin and mianserin. The basal level of phosphate incorporation into Protein I in the absence of serotonin and mianserin was 6.7 ± 0.4 pmol per mg Zn acetate-precipitable protein. Data represent the mean $\pm$ s.e.m. for five samples, each assayed in duplicate. *, $P < 0.01$ when the effect of serotonin is compared with basal level (Student's *t*-test).

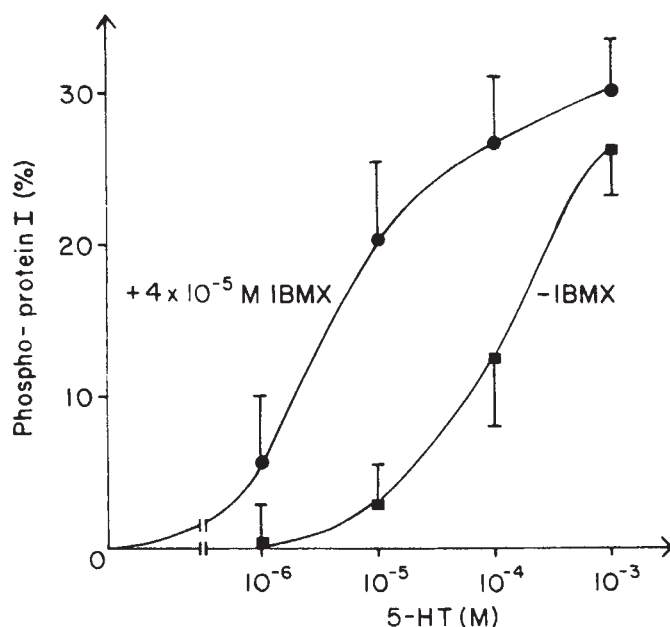


Fig. 2 Formation of phospho-Protein I from dephospho-Protein I in slices of rat facial nucleus incubated in the presence of various concentrations of serotonin. Experimental slices were incubated in the presence of the indicated concentration of serotonin, in the presence (●) or absence (■) of 4×10^{-5} M IBMX. Paired control slices were incubated in the absence of serotonin, in the presence or absence of 4×10^{-5} M IBMX, respectively. After termination of the incubation and extraction of Protein I from the slices, the amount of Protein I present in the dephospho-form in the extract was assayed by back-phosphorylation of dephospho-peptide 1, as described in Fig. 1 legend. The amount of phosphate incorporated into Protein I was determined as previously described⁶, and the per cent conversion of dephospho- to phospho-Protein I due to serotonin was then calculated for each pair of incubations. Each data point represents the mean $\pm$ s.e.m. of 5–10 pairs of samples, each assayed in duplicate. The basal level of phosphate incorporation into Protein I was 7.9 ± 0.8 pmol per mg Zn acetate-precipitable protein ($n=25$) in the absence of IBMX, and 7.4 ± 0.7 pmol per mg Zn acetate-precipitable protein ($n=25$) in the presence of IBMX (4×10^{-5} M).

peptide 1 of Protein I in synaptosomes prepared from the rat facial nucleus: these results were obtained by preincubating synaptosomes with 32 P-inorganic phosphate to prelabel the presynaptic ATP pool, and then measuring the incorporation of 32 P into Protein I in response to incubation of the synaptosomes with serotonin²⁶.

In a previous study of the facial nucleus, using electron microscope autoradiography with 3 H-serotonin, no specialized axo-axonic synapses involving serotonergic terminals were seen¹³. All the serotonergic terminals observed to form synapses innervated motoneurone somata or dendrites¹³. Electrophysiological studies suggest that a major effect of the serotonin input to the facial nucleus is to facilitate motoneurone excitation by a direct action on the motoneurone cell bodies^{10,13,27}. The present studies on Protein I phosphorylation indicate that serotonin may also serve a paracrine function²⁸, diffusing to neighbouring presynaptic elements and possibly modifying neurotransmitter release. Thus, in the facial motor nucleus, serotonin may modulate function in both pre- and postsynaptic elements. Support for this interpretation is provided by studies of the crustacean neuromuscular junction, where serotonin acts both on excitatory nerve endings to facilitate transmitter release²⁹ and directly on muscle fibres to stimulate contracture³⁰.

We thank Dr G. K. Aghajanian for many helpful discussions and for provision of unpublished data. This work was supported by USPHS grants AA-04183, DA-01627, MH-17387 and NS-08440 and a grant from the McKnight Foundation.

Received 8 July; accepted 10 November 1980.

1. Ueda, T. & Greengard, P. *J. biol. Chem.* **252**, 5155–5163 (1977).
2. Ueda, T., Maeno, H. & Greengard, P. *J. biol. Chem.* **248**, 8295–8305 (1973).
3. Krueger, B. K., Forn, J. & Greengard, P. *J. biol. Chem.* **252**, 2764–2773 (1977).
4. Huttner, W. B. & Greengard, P. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5402–5406 (1979).
5. Bloom, F. E., Ueda, T., Battenberg, E. & Greengard, P. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5982–5986 (1979).
6. De Camilli, P., Ueda, T., Bloom, F. E., Battenberg, E. & Greengard, P. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5977–5981 (1979).

7. Ueda, T. et al. *J. Cell Biol.* **83**, 308–319 (1979).
8. Forn, J. & Greengard, P. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5195–5199 (1978).
9. Courville, J. *Brain Res.* **1**, 338–354 (1966).
10. McCall, R. B. & Aghajanian, G. K. *Brain Res.* **169**, 11–27 (1979).
11. Dahlström, A. & Fuxe, K. *Acta physiol. scand.* **64**, 1–36 (1965).
12. Palkovits, M., Brownstein, M. & Saavedra, J. M. *Brain Res.* **80**, 237–249 (1974).
13. Aghajanian, G. K. & McCall, R. B. *Neuroscience* (in the press).
14. Brown, B. L., Dolphin, A. C. & Greengard, P. (in preparation).
15. Greengard, P. *Harvey Lect.* (Academic, New York, 1981).
16. Reddington, M., Rodnight, R. & Williams, M. *Biochem J.* **132**, 475–482 (1973).
17. De Blas, A. L., Wang, Y. J., Sorensen, R. & Mahler, H. R. *J. Neurochem.* **33**, 647–659 (1979).
18. Ng, M. & Matus, A. *Neuroscience* **4**, 169–180 (1979).
19. Zwiers, H., Tonnaer, J., Wiegant, V. M., Schotman, P. & Gispen, W. H. *J. Neurochem.* **33**, 247–256 (1979).
20. Lohmann, S. M., Walter, U. & Greengard, P. *J. biol. Chem.* (in the press).
21. Vargaftig, B. B., Coignet, J. L., De Vos, C. J., Grijsen, H. & Bonta, I. L. *Eur. J. Pharmac.* **16**, 336–346 (1971).
22. Nelson, D. L., Herbert, A., Bourgois, S., Glowinski, J. & Hamon, M. *Molec. Pharmac.* **14**, 983–995 (1978).
23. Enjalbert, A., Hamon, M., Bourgois, S. & Bockaert, J. *Molec. Pharmac.* **14**, 11–23 (1978).
24. Swanson, L. W. & Hartman, B. K. *J. comp. Neurol.* **163**, 467–506 (1975).
25. Dolphin, A. C. & Greengard, P. *J. Neurosci.* (in the press).
26. Whitman, M., Dolphin, A. C., Huttner, W. B. & Greengard, P. (in preparation).
27. McCall, R. B. & Aghajanian, G. K. *Neuroscience* **4**, 1501–1510 (1979).
28. Reader, T. A., Ferron, A., Descarries, L. & Jasper, H. H. *Brain Res.* **160**, 217–229 (1979).
29. Dudel, J. *Naunyn-Schmiedeberg's Archs exp. Path. Pharmac.* **249**, 515–528 (1965).
30. Kravitz, E. A., Battelle, B.-A., Evans, P. D., Talemo, B. R. & Wallace, B. G. *Neurosci. Symp.* **1**, 67–81 (1975).
31. Bradford, M. M. *Analyt. Biochem.* **72**, 248–254 (1976).
32. Beavo, J. A., Bechtel, P. J. & Krebs, E. G. *Meth. Enzym.* **38C**, 299–309 (1974).
33. Huttner, W. B., DeGennaro, L. J. & Greengard, P. *J. biol. Chem.* (in the press).

Defect in cyclic AMP phosphodiesterase due to the *dunce* mutation of learning in *Drosophila melanogaster*

Duncan Byers*

Division of Biology, California Institute of Technology, Pasadena, California 91125

Ronald L. Davis* & John A. Kiger Jr

Department of Genetics, University of California at Davis, Davis, California 95616

Cyclic AMP is an intracellular mediator ('second messenger') in the nervous and endocrine control of cellular function, regulating different processes in different cell types. Although evidence is incomplete, it seems that cyclic AMP enhances the calcium-mediated release of neurotransmitter in some neurones^{1–3}. A simple form of memory in the mollusc *Aplysia* is probably encoded as a cyclic AMP-induced enhancement of neurotransmission at certain synapses of the central nervous system⁴. The possibility that cyclic AMP participates in learning mechanisms may be explored using genetic mutants. For this purpose the fruitfly *Drosophila* is suitable as it is genetically well characterized and can learn through olfaction⁵, vision⁶ or taste⁷. We show here that independent searches for mutations of olfactory learning⁸ and of cyclic AMP metabolism⁹, and for mutations causing female infertility¹⁰ have each led to the same gene—the *dunce* gene. Our evidence indicates that the normal *dunce* gene may specify a cyclic AMP phosphodiesterase.

Two different mutant alleles of the *dunce* gene, *dunce*¹ and *dunce*², were isolated in a search for mutations of olfactory learning⁸. The *dunce* mutants do not learn to avoid an odorant associated with electric shock or quinine powder, although both of these are effective reinforcers for normal flies. The mutants can sense odorants, however, as they show normal inherent olfactory preferences in other situations^{8,11}. The learning performance of *dunce* in other sorts of learning test can be normal (visual learning¹²), intermediate (leg-position learning; R. Booker and W. G. Quinn, personal communication) or poor (larval olfactory learning¹³). In morphology and general vigour, *dunce*¹ and *dunce*² seem normal. Both mutations reduce the fecundity of females, *dunce*¹ to about half the normal number of offspring and *dunce*² to 2% of the normal¹⁴.

Table 1 Learning in the *dunce* mutants

Genotype	Olfactory learning index (Λ)
+ (normal Canton-S)	0.33 $\pm$ 0.01 (n = 463)
<i>dunce</i> ¹	0.04 $\pm$ 0.01 (n = 88)
<i>dunce</i> ²	0.05 $\pm$ 0.01 (n = 47)
<i>Df(1)N</i> ^{71h24-5} / <i>Df(1)dm</i> ^{75e19}	0.03 $\pm$ 0.03 (n = 6)
<i>dunce</i> ¹ / <i>dunce</i> ²	0.07 $\pm$ 0.02 (n = 6)
<i>dunce</i> ¹ / <i>Df(1)N</i> ^{71h24-5}	0.05 $\pm$ 0.01 (n = 4)
<i>dunce</i> ² / <i>Df(1)N</i> ^{71h24-5}	0.02 $\pm$ 0.02 (n = 6)
<i>dunce</i> ² / <i>Df(1)dm</i> ^{75e19}	0.02 $\pm$ 0.02 (n = 5)
+/ <i>dunce</i> ¹	0.26 $\pm$ 0.02 (n = 9)
+/ <i>dunce</i> ²	0.25 $\pm$ 0.05 (n = 9)
+/ <i>Df(1)N</i> ^{71h24-5}	0.18 $\pm$ 0.03 (n = 5)
+/ <i>Df(1)dm</i> ^{75e19}	0.26 $\pm$ 0.03 (n = 5)

Scores are mean $\pm$ s.e.m. of n independent determinations of the learning index, Λ . The mutants *dunce*¹ and *dunce*² were previously referred to as *dunce*^{DB38} and *dunce*^{DB276} respectively^{11–13}. The learning tests of normal (+), *dunce*¹ and *dunce*² were made with males alone, females alone and mixed groups; there were no significant sexual differences in learning. The other genetic combinations were tested as females heterozygous for two different X chromosomes, listed one before and one after a solidus. Before learning was tested, the two deficiency strains were backcrossed for two generations with strains derived from Canton-S to reduce possible genetic variation due to chromosomes other than the X. For learning measurements, flies were trained and tested in groups as previously described^{5,8}, with 3-octanol and 4-methylcyclohexanol as odorants and electric shock as punishment (see text). The learning index, Λ , is defined as the fraction of flies avoiding the odorant previously associated with shock minus the fraction avoiding the control odorant⁵. A positive score indicates learning (theoretical maximum = 1.0) and zero indicates no learning.

Other mutations of the *dunce* gene and some genetic deletions of *dunce* were identified through study of the genetics of cyclic AMP metabolism in *Drosophila*. As in other animals, cyclic AMP is synthesized from ATP by adenylyl cyclase and hydrolysed to 5'-AMP by cyclic AMP phosphodiesterase. Animals typically have several forms (isozymes) of cyclic AMP phosphodiesterase^{15–17}. In *Drosophila*, two forms, here designated I and II, have been described and partially purified; these differ in molecular weight, substrate specificity and other properties^{18,19}. A search for genes with major control over the cyclic AMP phosphodiesterase activity revealed that the 'genetic dosage' of region 3D3–3D4 of the X chromosome affects the activity of the smaller form (form II) in a manner suggesting that this region may include the structural gene of form II^{9,18}. Using two genetic deletions, *Df(1)N*^{71h24-5} and *Df(1)dm*^{75e19} (see Fig. 1), one can generate (female) flies not carrying this region on either X chromosome²⁰. These females are morphologically

Table 2 Cyclic AMP phosphodiesterase activity in unfractionated homogenates of the *dunce* mutants

Genotype	Cyclic AMP phosphodiesterase activity (pmol cyclic AMP hydrolysed per min per mg live weight)	
	Male	Female
Normal (Canton-S)	226 $\pm$ 7 (n = 10)	174 $\pm$ 8 (n = 11)
<i>dunce</i> ¹	135 $\pm$ 13 (n = 6)	113 $\pm$ 10 (n = 6)
<i>dunce</i> ²	94 $\pm$ 13 (n = 6)	101 $\pm$ 5 (n = 6)

Activities are mean $\pm$ s.e.m. of n assays using the procedure of Kiger and Golanty⁹. For each assay two flies were homogenized at 0°C in 0.4–1.0 ml of buffer. Duplicate 0.05-ml portions of each homogenate were mixed with 0.05 ml of tritium-labelled cyclic AMP in water, yielding reaction mixtures containing 10⁻⁴ M cyclic AMP, 10 mM MgCl₂, 2 mM 2-mercaptoethanol and 40 mM Tris-HCl, pH 8.0. The mixtures were incubated at 30°C for 20 min and then heated at 90°C to stop the reactions. Substrate and products were separated by chromatography on paper, eluted from the paper and measured in a scintillation counter.

* Present addresses: European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69 Heidelberg, FRG (D.B.); Department of Chemistry, California Institute of Technology, Pasadena, California 91125 (R.L.D.).

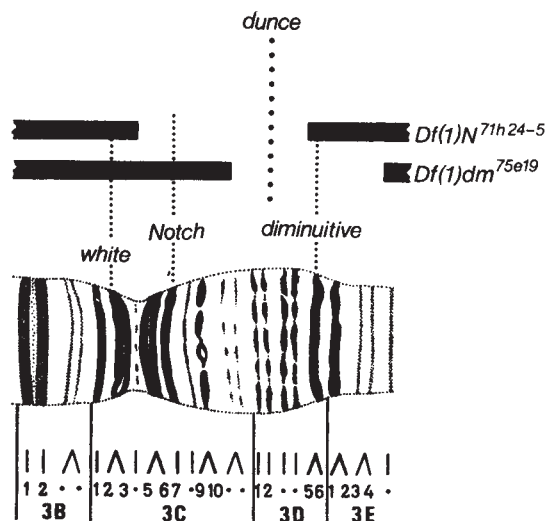


Fig. 1 Cytogenetic location of the *dunce* gene and two deletions of the *dunce* gene. At the top are illustrated the breakpoints found by Lefevre of Deficiency (first chromosome) *Notch*^{71h24-5} (*Df(1)N*^{71h24-5}), a deletion of chromosomal bands 3C4 to 3D4 inclusive, and *Df(1)diminutive*^{75e19} (*Df(1)dm*^{75e19}), a deletion of bands 3C12 to 3E4 inclusive²⁷. The horizontal solid bars indicate regions of intact chromosome and the gaps indicate deleted regions. In alignment below is part (3%) of Bridges' drawing²⁸ of the *Drosophila* X chromosome of the larval salivary cells. Locations of the genes *white*, *Notch* and *diminutive* are also shown.

normal and appear to be vigorous and healthy, even though they lack five adjacent chromosomal bands, including the *dunce* locus. They are sterile, and they lack the form II of cyclic AMP phosphodiesterase activity.

Still two other mutant alleles of the *dunce* gene have been found among 225 female-sterile mutants isolated by Mohler¹⁰ on the *Drosophila* X chromosome. We show no data for them here but introduce them briefly. Both are located within chromosomal region 3C12–3D4 and are deficient in the form II of cyclic AMP phosphodiesterase; this was the first evidence that female infertility and defective cyclic AMP metabolism are associated with one gene in this region rather than two neighbouring genes²¹. In addition, the two mutants of Mohler are unsuccessful in olfactory learning and fail to complement *dunce*² in learning or fertility¹⁴. Therefore they are alleles of the *dunce* gene, and we, with Mohler, rename them *dunce*^{M11} and *dunce*^{M14}.

Measurements of olfactory learning ability were made using the olfactory learning paradigm of Quinn, Harris and Benzer³. For training, groups of approximately 50 flies were attracted

with a light to run alternately on to two printed circuit grids spread with different odorants, with one of the grids electrified. To test later whether learning had occurred, similar grids were used without shock, and the number of flies avoiding each odorant grid recorded. Normal and *dunce* flies tend to avoid the charged grid during training. Afterwards, however, normal flies selectively avoid the odorant previously paired with shock, whereas *dunce* flies do not⁸. Normal flies can learn to avoid the odorant on either of the two training grids, depending only on which grid is electrified: therefore the selective avoidance is not due to pseudoconditioning or inherent odour bias, but is an example of associative learning³. The learning index, Λ , defined in Table 1 legend, is a measure of the selective avoidance⁵.

For normal flies of the Canton-S strain, Λ is 0.33 (Table 1). The mutants *dunce*¹, *dunce*² and *Df(1)N*^{71h24-5}/*Df(1)dm*^{75e19} learn poorly ($\Lambda = 0.04$, 0.05 and 0.03, respectively). Heterozygous *dunce*/+ flies do not seem to learn as well as +/+ normal flies, suggesting that one normal gene may not be sufficient for normal learning. Flies heterozygous for pairs of different *dunce* deletions or mutations learn poorly (Table 1), showing that the different mutations do not complement each other in the combinations tested. Therefore, the *dunce* gene is located within the region of overlap of the two deletions.

The cyclic AMP phosphodiesterase activity of *dunce* was measured in two ways. First, adult flies were homogenized, radio-labelled cyclic AMP was added and the rate of hydrolysis of cyclic AMP measured. The rate of hydrolysis of cyclic AMP is reduced in *dunce*¹ and *dunce*² to approximately half of normal (Table 2). Second, groups of normal or *dunce* flies were homogenized, then centrifuged briefly to remove particulates, the supernatants were fractionated on sucrose density gradients and the distribution of cyclic AMP phosphodiesterase activity on the gradients measured. Normal *Drosophila* display two prominent forms (I and II) of soluble cyclic AMP phosphodiesterase¹⁸, as shown in Fig. 2. The mutants *dunce*¹ and *dunce*² seem to have

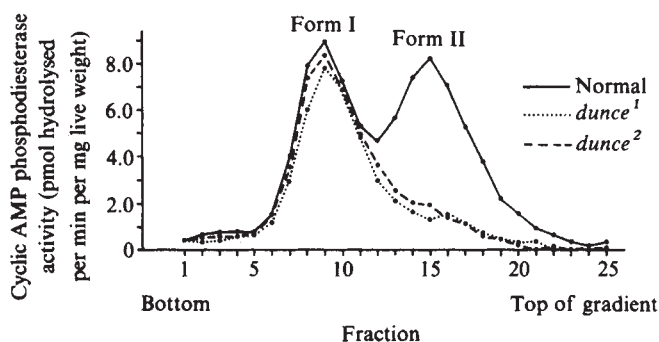


Fig. 2 Sucrose density gradient sedimentation of the soluble cyclic AMP phosphodiesterase activity of normal (Canton-S) and *dunce* *Drosophila*. Enzyme activity is expressed in pmol of cyclic AMP hydrolysed per min per mg live weight. Groups of 40 adult females were homogenized at 0°C in 1.0 ml of buffer (10 mM MgCl₂, 1 mM CaCl₂, 2 mM 2-mercaptoethanol, and 40 mM Tris-HCl, pH 8.0). The same buffer was used for all subsequent steps. The homogenates were centrifuged at 105,000g for 60 min. 0.2-ml portions of the supernatants were layered on 5–20% sucrose density gradients (4.6 ml) and centrifuged at 165,000g (r_{av}) for 18 h. The gradients were divided into 0.18-ml fractions and duplicate 0.08-ml portions of each fraction were mixed with 0.02 ml of 5×10^{-4} M tritium-labelled cyclic AMP (NEN), incubated at 30°C for 30 min, and then heated at 90°C to stop the reactions. The product, 5'-AMP, was converted to adenosine with unpurified *Crotalus atrox* venom (Sigma) and measured with the resin assay of Thompson and Appleman¹⁵. Blank controls were treated identically except that buffer was added instead of homogenate; the radioactivity of the blanks (4,100 c.p.m.) was subtracted from each fraction. Before correction, the most active fractions contained about 15,000 c.p.m., representing hydrolysis of 12% of the radioactive cyclic AMP. The recovery of applied cyclic AMP phosphodiesterase activity from each gradient was ~50%.

Table 3 Cyclic AMP in the *dunce* mutants

Genotype	Cyclic AMP in adult whole fly (pmol per mg live weight)	
	Male	Female
Normal (Canton-S)	1.5 ± 0.1 (n = 4)	1.6 ± 0.1 (n = 6)
<i>dunce</i> ¹	2.7 ± 0.1 (n = 6)	2.3 ± 0.2 (n = 6)
<i>dunce</i> ²	2.8 ± 0.1 (n = 6)	2.6 ± 0.1 (n = 5)

Cyclic AMP was measured by radioimmunoassay (NEN) in which *Drosophila* cyclic AMP competed with a standard amount of ¹²⁵I-labelled cyclic AMP for antibody binding sites. Values are mean ± s.e.m. of *n* extracts assayed. For each extract, four to six flies were homogenized together in 0.1 M HCl at room temperature. The extracts were heated for 2 min in a boiling water bath, cleared by centrifugation, neutralized to pH 6.2, cleared again by centrifugation, diluted and assayed as described previously²². The recovery of cyclic AMP was estimated with a small quantity of tritium-labelled cyclic AMP added to the initial homogenates and averaged 96%. If the cyclic AMP of normal flies (1.5 pmol per mg) were distributed uniformly through the fly, its concentration would be ~1.5 μM.

normal amounts of form I and their activity at the position of form II is small, though not zero (Fig. 2). On similar gradients of *dunce*^{M11}, *dunce*^{M14} and *Df(1)N*^{71h24-5}/*Df(1)dm*^{75e19} (not shown), form I is approximately normal and form II is undetectable (less than 5% of normal).

If the activity of a cyclic AMP phosphodiesterase were reduced, then the concentration of cyclic AMP in the fly might be expected to rise. To test this, the amount of cyclic AMP in normal and *dunce* flies was measured using a radioimmunoassay. Normal adults yielded 1.5 pmol of cyclic AMP per mg live weight (Table 3 and ref. 22). The mutants *dunce*¹ and *dunce*² yielded about 160% of the normal quantity of cyclic AMP (Table 3). Cyclic AMP in similar extracts of *dunce*^{M11}, *dunce*^{M14} and *Df(1)N*^{71h24-5}/*Df(1)dm*^{75e19} is 300–600% of the normal^{21,22}.

We conclude that the normal *dunce* gene product has roles in behaviour, reproduction and cyclic AMP metabolism. The results show that the *dunce* gene and one isozyme of cyclic AMP phosphodiesterase are closely related in some way, but further

experiments are required to test whether *dunce* is the structural gene of this enzyme. If elimination of cyclic AMP phosphodiesterase is in fact the cause of poor learning in *dunce*, then pharmacological inhibition of cyclic AMP phosphodiesterase activity should interfere with learning in normal flies. This expectation is confirmed by an experiment of Dudai and Jan²³, recently reconfirmed by Dudai²⁴. They found that the olfactory learning performance of *dunce* mutants is mimicked by normal flies fed caffeine, an inhibitor of cyclic nucleotide phosphodiesterases^{25,26}. One question still unanswered is whether the normal product of the *dunce* gene forms an integral part of a molecular apparatus of learning.

We thank J. D. Mohler and G. Lefevre Jr for mutant strains. D.B. thanks Seymour Benzer for support and laboratory facilities, and Lawrence Kauvar, Sandra Shotwell and Shinobu Fujita for biochemical advice. Financial support was from the NSF (to S. Benzer) and NIH (to J.A.K.); D.B. was a fellow of the Gordon Ross Medical Foundation and R.L.D. was a NIH Pre-Doctoral Trainee.

Received 22 July; accepted 25 September 1980.

- Shimihara, T. & Tauc, L. *Brain Res.* **127**, 168–172 (1977).
- O'Shea, M. & Evans, P. D. *J. exp. Biol.* **79**, 169–190 (1979).
- Goldberg, A. L. & Singer, J. J. *Proc. natn. Acad. Sci. U.S.A.* **64**, 134–141 (1969).
- Klein, M. & Kandel, E. R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3512–3516 (1978).
- Quinn, W. G., Harris, W. A. & Benzer, S. *Proc. natn. Acad. Sci. U.S.A.* **71**, 708–712 (1974).
- Menne, D. & Spatz, H. C. *J. comp. Physiol.* **114**, 301–312 (1977).
- Medioni, J. & Vayssie, G. C. *r. séanc. Soc. Biol.* **169**, 1386–1391 (1975).
- Dudai, Y., Jan, Y., -N., Byers, D., Quinn, W. G. & Benzer, S. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1684–1688 (1976).
- Kiger, J. A. Jr & Golanty, E. *Genetics* **85**, 609–622 (1977).
- Mohler, J. D. *Genetics* **74**, s184 (1973); **85**, 259–272 (1977).
- Dudai, Y. *J. comp. Physiol.* **130**, 271–275 (1979).
- Dudai, Y. & Bicker, G. *Naturwissenschaften* **65**, 494–495 (1978).
- Aceves-Piña, E. O. & Quinn, W. G. *Science* **206**, 93–96 (1979).
- Byers, D. thesis, California Inst. Technol. (1979).

- Thompson, W. J. & Appleman, M. M. *Biochemistry* **10**, 311–316 (1971).
- Van Imwegen, R. G., Pledger, W. J., Strada, S. J. & Thompson, W. J. *Archs Biochem. Biophys.* **175**, 700–709 (1976).
- Weiss, B. *Adv. Cyclic Nucleotide Res.* **5**, 195–211 (1975).
- Kiger, J. A. Jr & Golanty, E. *Genetics* **91**, 521–535 (1979).
- Davis, R. L. & Kiger, J. A. Jr *Archs Biochem. Biophys.* **203**, 412–421 (1980).
- Kiger, J. A. Jr *Genetics* **85**, 623–628 (1977).
- Davis, R. L. thesis, Univ. California (1979).
- Davis, R. L. & Kiger, J. A. Jr *Biochem. biophys. Res. Commun.* **81**, 1180–1186 (1978).
- Dudai, Y. & Jan, Y., -N. *Biol. a. Rep.*, California Inst. Technol., 72 (1975).
- Dudai, Y. *Abstr. a. Mtg. Isr. biochem. Soc.*, 100 (1979).
- Sutherland, E. W. & Rall, T. W. *J. biol. Chem.* **232**, 1077–1091 (1958).
- Goodman, L. S. & Gilman, A. (eds) in *Pharmacological Basis of Therapeutics* 5th edn, 367–378 (Macmillan, New York, 1975).
- Craymer, L. & Roy, E. *Drosoph. Inf. Serv.* **55**, 200–204 (1980).
- Bridges, C. B. *J. Hered.* **29**, 11–13 (1938).

Altered substrate specificity of herpes simplex virus thymidine kinase confers acyclovir-resistance

G. Darby & H. J. Field

Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 2QQ, UK

S. A. Salisbury

Department of Organic and Inorganic Chemistry, University of Cambridge, Cambridge, UK

Acyclovir (9-[2-hydroxyethoxymethyl]guanine or ACV) is a nucleoside analogue with considerable potential for the treatment of herpes simplex virus (HSV) infections in man^{1,2}. Two virus-coded enzymes are important in the mechanism of action of this drug: thymidine kinase (TK) which initiates its activation by converting it to the monophosphate³ and DNA polymerase whose action is inhibited by ACV triphosphate⁴. Changes in either gene may confer resistance^{5–7}, but all reported mutations in the TK gene have resulted in failure of the resistant virus to induce appreciable levels of the enzyme. Such TK[–] mutants arise readily in tissue culture systems^{8,9} where the enzyme is non-essential for virus replication¹⁰, but in animals they show considerably reduced pathogenicity and neurovirulence^{7,11–14}. We now describe the isolation of a resistant mutant which induces a TK of altered substrate specificity and we show that this virus retains pathogenicity for mice with only a slight attenuation of neurovirulence.

To obtain conditions *in vitro* which would be selective against the readily occurring TK[–] variants, serum-starved BHK cells were used. It is known that TK[–] virus is at a disadvantage in resting cells^{11,15} and it was therefore hoped that selecting for resistance in these cells would increase the probability of isolating TK⁺ resistant virus. In fact both TK⁺ and TK[–] resistant clones were obtained using this method.

Table 1 Resistance of TK⁺ and TK[–] variants of SC16

Virus	TK (% SC16)	Resistance to ACV (ED ₅₀ , µg ml ^{–1})		Resistance to PAA (ED ₅₀ , µg ml ^{–1}) BHK cells
		BHK cells	D21 cells*	
SC16	100	0.02	0.01	11
SC16 S1	30–40	2.7	0.01	12
SC16 S6	<0.1	3.0	ND	ND

The parental strain of HSV1 (SC16) was originally isolated from an oral lesion. It has been well characterized in mice²¹ and has previously been used to derive ACV-resistant variants⁷. 2×10^6 BHK cells were seeded into 25-cm² plastic tissue-culture flasks and incubated overnight in Glasgow-modified Eagle's medium supplemented with 10% tryptose phosphate broth and 10% calf serum. After 24 h the medium was removed, the cells washed and the flasks replenished with Eagle's medium without tryptose phosphate broth and with calf serum reduced to 0.5%. The cells were then maintained for 5 d before being inoculated with SC16 at a multiplicity of 0.1 plaque-forming unit (PFU) per cell in the presence of 0.1 µg ml^{–1} ACV. After 3 d of incubation, the cells were collected, subjected to ultrasonic disruption, then the released virus was titrated and used to inoculate further bottles of serum-starved cells. The second and third passage were in 1.0 and 10 µg ml^{–1} ACV respectively. Clones were isolated from the virus yield from the third passage. TK activity was measured on extracts of BUdR-resistant BHK (BU-BHK) cells obtained 18 h after inoculation with virus at 5 PFU per cell. The cells were resuspended at 3×10^7 per ml in 0.01M Tris and disrupted by ultrasonic vibration. Reactions were performed at 37 °C in 0.125-ml aliquots containing 0.02M phosphate buffer pH 6, 5mM MgCl₂, 5mM ATP and 40 µM 14C-thymidine (1.6 µCi ml^{–1}). Reactions were carried out using the method described by Klemperer *et al.*²². The results are expressed as % phosphorylation compared with the SC16 extract. Plaque reduction assays for both ACV and PAA were performed by inoculating monolayers of BHK cells with 200 PFU virus. After adsorption for 1 h, overlay medium was added containing increasing concentrations of the test drug. Graphs were plotted of % plaque reduction against log₁₀ drug concentration and the ED₅₀ values were assessed directly from the graphs.

* D21 cells are LTK[–] cells transformed to TK⁺ phenotype using sheared HSV-2 DNA²³.

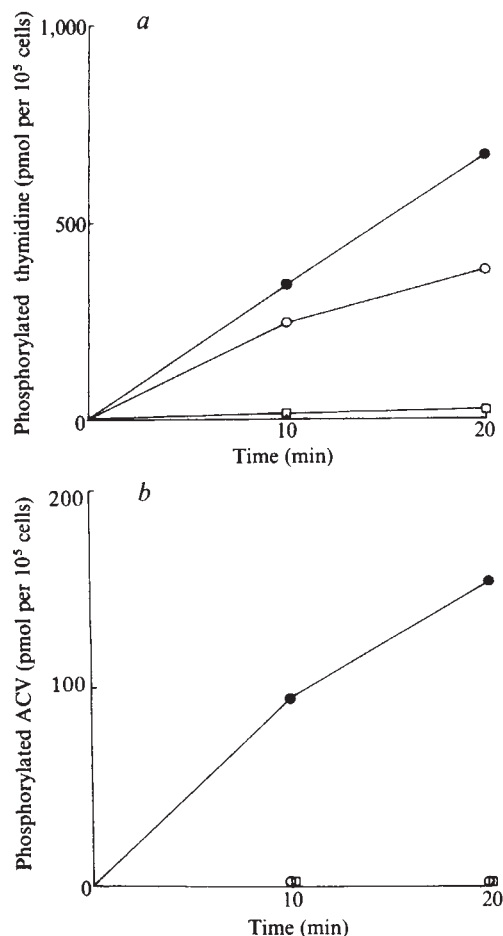


Fig. 1 The phosphorylation of thymidine and acyclovir by extracts of BU BHK cells infected with wild-type or ACV-resistant HSV. *a*, Phosphorylation of thymidine; *b*, phosphorylation of acyclovir. ●, SC16; ○, SC16 S1; □, SC16 S6. The preparation of infected cell extracts and assay for thymidine kinase are described in Table 1 legend. The assay for ACV was similar except that ^{14}C -thymidine was replaced by $0.8\text{ mM } ^3\text{H-ACV}$ ($40\text{ }\mu\text{Ci ml}^{-1}$). The reactions were allowed to proceed for 0, 10 and 20 min and the 0-min values were subtracted. $^3\text{H-ACV}$ was prepared by a modification of the procedure described by Shelton and Clark²⁴.

HSV-1 strain SC16 was passed in the presence of increasing concentrations of ACV in serum-starved BHK cells and the yield from the third passage was cloned in the absence of drug in actively growing BHK cells. Two of these clones (SC16 S1 and SC16 S6) were examined in detail. Both were shown to be approximately 100-fold more resistant to ACV (ED_{50} $2\text{--}3\text{ }\mu\text{g ml}^{-1}$) than the parental strain (Table 1). Thymidine kinase assays on infected cell extracts taken 18 h after high multiplicity infection showed that SC16 S1 induced TK whereas no activity was detected in the case of S6. However, the level of TK induced by SC16 S1 was rather less (30–40%) than that induced by the parental virus. One possible explanation for this difference would be a mutation in the TK gene.

Three lines of evidence were subsequently obtained which also suggested that the resistance of SC16 S1 resides in an altered TK and not at the level of DNA polymerase. First, SC16 S1 was sensitive to ACV when tested in D₂₁ cells which carry and express the HSV TK gene (Table 1). These cells 'activate' the drug and only viruses which have acquired resistance at loci other than TK are resistant in them^{8,16}. Secondly, viruses which are resistant at the DNA polymerase locus are also frequently resistant to phosphonoacetic acid (PAA)^{5,6,8}. However, SC16 S1 was as sensitive as the parent to this drug (Table 1).

The third line of evidence concerns the direct measurement of the ability of the induced TK to phosphorylate ACV *in vitro*. The ability of extracts from cells infected with SC16 or ACV-resistant strains to phosphorylate thymidine and ACV were compared. The results of this experiment are shown in Fig. 1. Clearly the parent virus is able to phosphorylate both thymidine

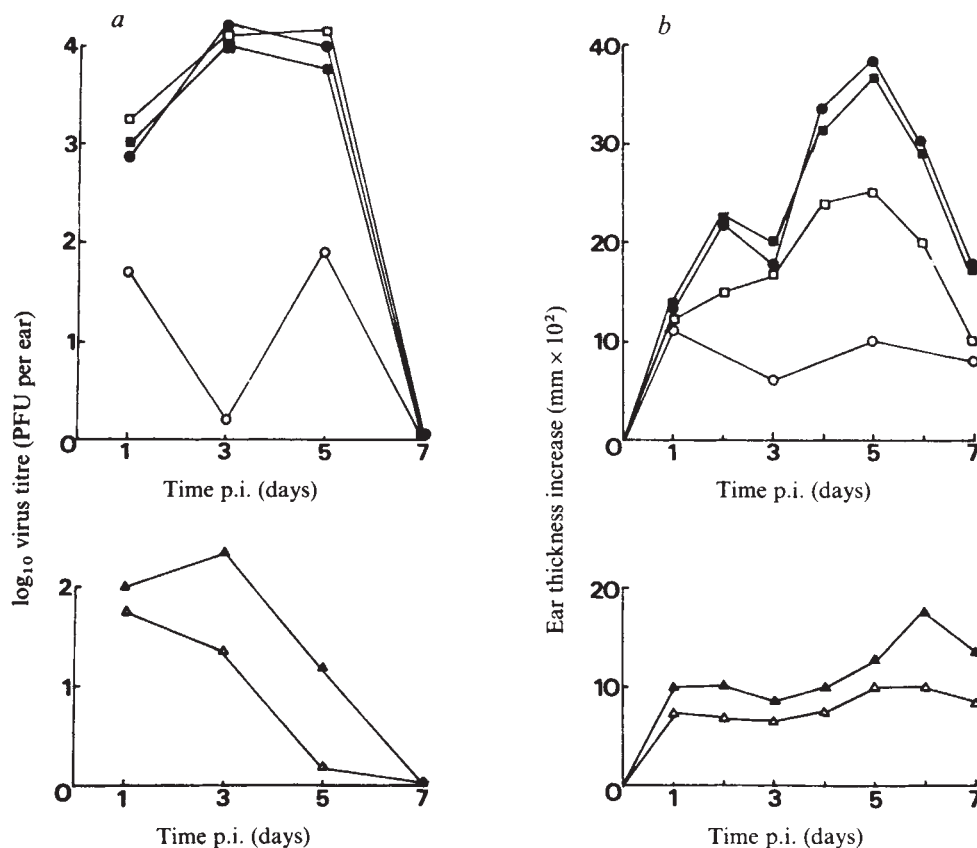


Fig. 2 Pathogenicity for mice of wild-type and TK⁺- and TK⁻- resistant strains of HSV, with and without ACV-treatment. *a*, Virus growth; *b*, cell-mediated inflammation. SC16: ●, untreated; ○, ACV-treated. SC16 S1: ■, untreated; □, ACV-treated. SC16 S6: ▲, untreated; △, ACV-treated. 10^4 PFU test virus were inoculated into the left ear pinna of anaesthetized, 4-week-old female BALB/c mice. Virus growth in the ear was measured by homogenizing the pinnae from groups of three mice, and virus titres were determined by plaque titration in BHK cells. Ear thickness was measured using an engineer's micrometer—the thickness increase has been found to be a reliable indicator of cell-mediated inflammation¹⁸. The drug was suspended in water and given in twice daily intraperitoneal injections totalling 50 mg per kg per day. The effects of ACV on sensitive and resistant strains of HSV in this model have been described previously^{7,17}. p.i., Post-infection.

Table 2 Virulence of TK⁺ and TK⁻ variants of SC16 in BALB/c mice

Virus	TK induction	Ear inoculation latency in cervical ganglia*	i.c. Inoculation PFU/LD ₅₀ [†]
SC16	+	7/10 (0/10)	6
SC16 S1	+	5/12	60
SC16 S6	-	0/12	>10 ⁶

* These mice are the survivors from the experiment illustrated in Fig. 2. The second, third and fourth cervical ganglia were explanted into Eagle's medium and incubated at 37°C for 6 d. The tissue was then homogenized and tested for the presence of infectious virus by plaque titration in BHK cells as described previously¹¹. Ratios are the number of mice from which latent virus was reactivated over the number of animals tested, the number in parentheses being the proportion positive following twice daily treatment with 50 mg per kg per day ACV injected intraperitoneally from the time of virus inoculation until 10 d thereafter.

[†] Dilutions of virus were inoculated into the left cerebral hemisphere in 0.02 ml Eagle's medium. The ED₅₀ value was obtained using the Spearman-Kärber method from the numbers of mice dying between days 2 and 14 after inoculation.

and ACV, though the actual amount of ACV phosphorylated is very much less. In contrast, the mutant-infected cell extracts failed to phosphorylate ACV although the S1 extract contained significant TK activity. Another isolate of HSV [C1(101)] which induces similar levels of TK to SC16 S1 was also tested; in this case ACV-phosphorylating activity was readily measured²⁰. All the evidence is therefore consistent with the resistance of SC16 S1 residing in its failure to activate the drug in infected cells and this in turn is presumably due to a mutation in the TK gene which generates an enzyme with an altered substrate specificity.

As the resistant virus SC16 S1 was TK⁺, it was obviously of interest to investigate its virulence in an animal model system. We have previously used the mouse ear model to investigate the pathogenicity of mutant viruses^{7,11}. It can be seen from the data (Fig. 2) that SC16 S1 grows as well in the mouse ear as does the parent strain. In contrast, the growth in the ear of the TK⁻ mutant SC16 S6 was greatly impaired. Furthermore, a normally effective treatment routine^{7,17} had little inhibitory effect on this virus because there was no marked reduction in virus titres in ear tissue or inflammation as determined by measurement of ear thickness¹⁸. The cervical dorsal root ganglia were later explanted from surviving mice to test for the presence of a latent infection (Table 2). Latent infections were detected in ganglion explants from both SC16 and SC16 S1 but not from S6. This was to be expected because of the reduced replication of the latter virus in the skin during the acute phase of the infection.

A further measure of the neurovirulence of the viruses was obtained by measuring the ED₅₀ dose following intracerebral inoculation^{11,19}. SC16 S1 seemed to be slightly attenuated relative to its parent showing a 10-fold increase in the ratio of plaque-forming units per LD₅₀ (Table 2). However, the TK⁻ strain, SC16 S6, was highly attenuated showing >1,000-fold increase. The pathogenicity data reported here are very similar to those obtained with another TK⁺ ACV-resistant strain of HSV [C1(101)P₂C₅]⁷, though recent evidence regarding this virus strongly suggests that, unlike SC16 S1, its resistance resides at the level of DNA polymerase and not TK²⁰.

The results described here suggest that HSV may acquire resistance to ACV by a mutation in the TK gene which alters the substrate specificity of the induced enzyme. Both this kind of mutant and the TK⁺ DNA-polymerase mutants which have already been reported would seem likely to arise *in vivo* as there is little reduction in their growth potential. It remains to be seen, however, whether such viruses will be isolated from or constitute a problem in patients undergoing treatment with nucleoside analogues such as ACV.

This work was supported by a MRC programme grant. We thank Mrs Elizabeth Lay for her technical assistance and Mrs M. Wright for preparing the manuscript. Acyclovir was a gift from Dr G. B. Elion.

Received 14 August, accepted 30 October 1980.

- Elion, G. B. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 5716-5720 (1977).
- Schaeffer, H. J. *et al. Nature* **272**, 583-585 (1978).
- Fyfe, J. A., Keller, P. M., Furman, P. A., Miller, R. L. & Elion, G. B. *J. biol. Chem.* **253**, 8721-8727 (1978).
- Furman, P. A. *et al. J. Virol.* **32**, 72-77 (1979).
- Coen, D. M. & Schaeffer, P. A. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2265-2269 (1980).
- Schnipper, L. E. & Crumpacker, C. S. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2270-2273 (1980).
- Field, H. J. & Darby, G. *Antimicrob. Ag. Chemother.* **17**, 209-216 (1980).
- Field, H. J., Darby, G. & Wildy, P. *J. gen. Virol.* **49**, 115-124 (1980).
- Smith, K. O., Kennell, W. L., Poirier, R. H. & Lynd, F. T. *Antimicrob. Ag. Chemother.* **17**, 144-150 (1980).
- Dubbs, D. R. & Kit, S. *Virology* **22**, 439-502 (1964).
- Field, H. J. & Wildy, P. *J. Hyg., Camb.* **81**, 267-277 (1978).
- Tenser, R. B. & Dunstan, M. E. *Virology* **99**, 417-422 (1979).
- Tenser, R. B., Miller, R. L. & Rapp, F. *Science* **205**, 915-917 (1979).
- Klein, R. J., De Stephano, E., Brady, E. & Friedman-Kien, A. E. *Arch. Virol.* (in the press).
- Jamieson, A. T., Gentry, G. A. & Subak-Sharpe, J. H. *J. gen. Virol.* **24**, 465-480 (1974).
- Darby, G., Larder, B. A., Bastow, K. F. & Field, H. J. *J. gen. Virol.* **48**, 451-454 (1980).
- Field, H. J., Bell, S. E., Elion, H. B., Nash, A. A. & Wildy, P. *Antimicrob. Ag. Chemother.* **15**, 554-561 (1979).
- Nash, A. A., Field, H. J. & Quartey-Papafio, R. *J. gen. Virol.* **48**, 351-364 (1980).
- Marcialis, M. A. *et al. Experimenta* **31**, 502-503 (1975).
- Field, H. J., McMillan, & Darby, G. *J. inf. Dis.* (in the press).
- Hill, T. J., Field, H. J. & Blyth, W. A. *J. gen. Virol.* **28**, 341-353 (1975).
- Klemperer, H. G., Haynes, G. R., Shedden, W. I. H. & Watson, D. H. *Virology* **31**, 502-503 (1967).
- Minson, A. C., Wildy, P., Buchan, A. & Darby, G. *Cell* **13**, 581-587 (1978).
- Shelton, K. R. & Clark, J. M. Jr. *Biochemistry* **6**, 2735-2739 (1967).

Terminal direct repeats in a retrovirus-like repeated mouse gene family

Eli Keshet & Yosef Shaul

Department of Virology, Weizmann Institute of Science, Rehovot, Israel

The mouse genome contains multiple copies of a dispersed gene family known individually as VL30 genes¹ which are thought to be associated with retroviruses. The copies consist of closely related 5.2-kilobase DNA sequences flanked by unrelated cellular DNA, and although no sequence homology has been found between the genes and the exogenous or endogenous retroviruses so far tested, the 30S RNA transcripts expressed by the genes are efficiently packaged into virions, recovered from infected cells²⁻⁴ and transmitted to other cells by pseudo-type infection⁵. Stimulated by recent reports⁶⁻¹⁰ of the similarity between retroviruses and transposons (from which retroviruses may have evolved⁶), and in particular by the recognition that both types of genetic elements are characterized by a large terminal repeat (LTR), we set out to determine whether the VL30 genes are also distinguished by this property. Using cloned DNAs from a mouse gene library and heteroduplex analysis, we have now found that the VL30 genes do indeed carry terminal direct repeats 400 base pairs long.

Individual copies of these 'retrovirus-like' genes (referred to as VL30 genes) were cloned from a gene library of the inbred mouse strain BALB/c in bacteriophage λ (ref. 1). The construction of the gene library from mouse genomic fragments obtained by a partial *EcoRI* digestion enabled us to select clones which, despite having *EcoRI* cleavages sites within VL30 DNA, encompass the entire 5.2 kilobases of the element and also contain some flanking DNA sequences. Such a clone with an *EcoRI* site, roughly in the middle of VL30 DNA, was used in the experiment designed to visualize LTRs (Fig. 1). As illustrated in Fig. 1, the presumptive LTRs are readily dissociated from each other by *EcoRI* digestion and subcloning of each fragment into the *EcoRI* site of plasmid pBR322. The subcloning procedure results in recombinant plasmids harbouring the eukaryotic insert in each of the two possible orientations. The orientation of insertion of several recombinant plasmids was determined by restriction enzyme digestions and blot hybridization analyses of the vector-insert junction fragments (data not shown). Two recombinant plasmids, whose VL30 DNA is inserted in the orientation depicted in Fig. 1, were linearized by *SalI* digestion and analysed by heteroduplexing. This procedure visualizes large repeats in VL30 DNA that are in a direct orientation. A

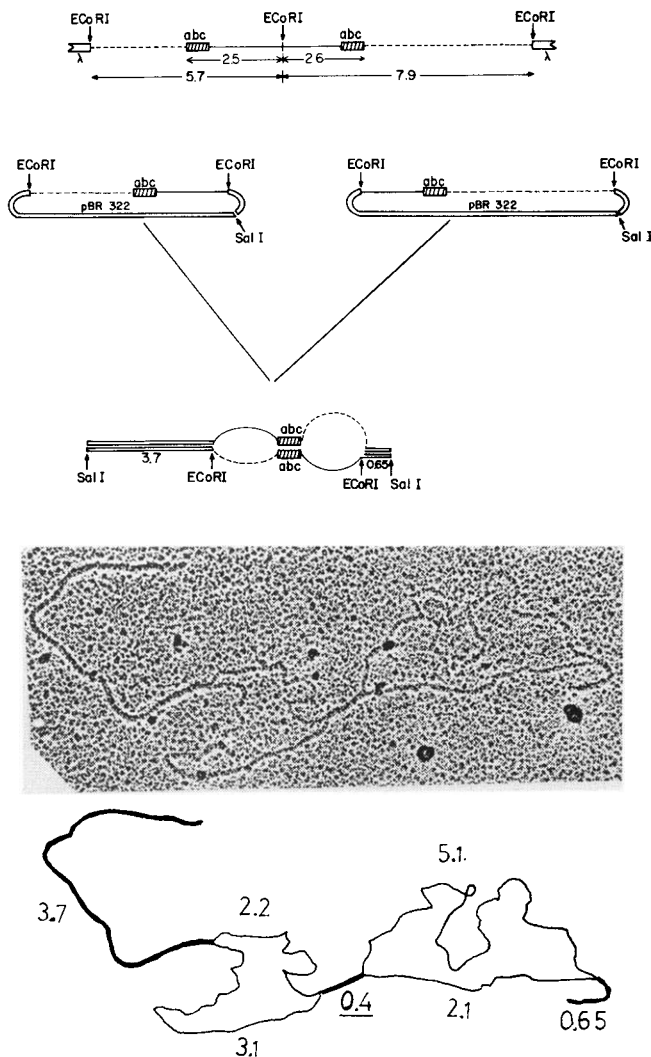


Fig. 1 Visualization of a direct repeat by heteroduplex analysis. Top: diagrammatic representation of cloned DNAs used and their expected heteroduplex. Upper drawing is of clone 3 of VL30 DNA in bacteriophage λ . Sizes (in kilobases) of the retrovirus-like DNA sequences (solid line) and their flanking DNA sequences (dashed line) were determined previously¹. Hatched boxes indicate presumptive direct repeats in DNA. The middle drawing is of subclones of the 5.7- and 7.9-kilobase *Eco*RI fragments of clone 3 in plasmid pBR322. The lower drawing is an expected heteroduplex structure between the two *Sal*I-linearized plasmids (drawing is out of scale). Bottom: electron micrograph of a typical heteroduplex. The indicated sizes (kilobases) are average values obtained from measurements of 10 heteroduplexes. The size of the duplexed region was standardized with respect to the duplexed regions of the vector (3.7 and 0.65 kilobases). The sizes of the single-stranded regions were standardized with respect to a single-stranded ϕ X174 included in the same field.

duplexed region, representing a direct repeat of ~ 400 base pairs, was observed (Fig. 1). A control heteroduplex, where the 5.7-kilobase *Eco*RI fragment is inserted into the plasmid in the opposite orientation (and is expected, therefore, to visualize inverted repeats) failed to reveal such a structure (data not shown). Several heteroduplexes were measured. On the basis of the known map location of the *Eco*RI site and the known sizes of the DNA sequences flanking this particular VL30 DNA¹, we could identify the direct repeat with the 400 base pairs residing at each terminus of VL30 DNA (Fig. 1).

As the inherent limitations in the accuracy of heteroduplex measurements prevent exact localization of the repeated DNA sequences within the cloned DNAs, we used another approach that exploits the availability of the poly(A)-containing 30S RNA

transcripts of VL30 DNA. A hybridization probe corresponding to the 3' end of the 30S RNA was prepared (Fig. 2). Although this probe contains sequences confined to the 3' end—one-fifth of the RNA—it efficiently hybridized to both *Eco*RI fragments (Fig. 2), thus confirming the existence of repeated DNA sequences, and ruling out the possibility that the repeats are contained within DNA sequences adjacent to VL30 DNA. Assuming a gross co-linearity between VL30 DNA and its 30S RNA transcript, this result also places the repeated sequences at both termini of VL30 DNA.

During the propagation of recombinant phages in bacteria, some of the cloned DNAs accumulated a significant fraction of phages with a shorter eukaryotic insert. Heteroduplex analysis revealed that all these phages suffered a unique deletion of 4.7 kilobases in VL30 DNA (data not shown) and suggested to us that the deletion mutants might have arisen as a result of intramolecular recombination involving the direct terminal repeats. Such an event would lead to the loss of all VL30 DNA sequences except for one copy of the LTR. To test this possibility, a deleted form of one of the cloned phages was heteroduplexed with non-deleted phage of another VL30 clone. The rationale for this experiment is given in Fig. 3. Two types of heteroduplex are anticipated which differ with respect to the choice of the LTR copy of the non-deleted phage that anneals with the sole LTR copy retained by the deleted phage. In each case, the two heteroduplexed DNAs shared only their λ 'arms' and an additional 0.4-kilobase segment (Fig. 3). The observed heteroduplexes were consistent with structures predicted on the basis of the known size of each flanking DNA sequence and thus verified the structure suggested for the deletion. It is likely that deletions, similar to those described above, that occur during

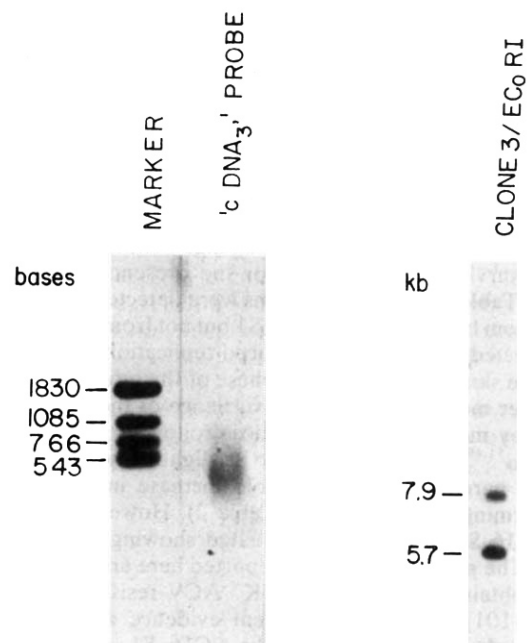


Fig. 2 Evidence for LTR in VL30 DNA with the aid of a 3' end-specific probe. 30S RNA was extracted from purified virions of Ra-MuLV produced by JLS-V9 cells. RNA was randomly sheared and poly(A)-containing RNA was purified by two cycles of oligo(dT)-cellulose chromatography. RNA was size fractionated by centrifugation through a sucrose gradient and molecules smaller than 800 bases were pooled. ³²P-labelled cDNA was synthesized with oligo(dT)₁₂₋₁₈ as a primer. This cDNA was designated cDNA_{3'}. Left: size determination of the cDNA_{3'} probe. A sample of cDNA_{3'} was electrophoresed through a 1.2% agarose gel and autoradiographed. Size markers are ³²P-labelled *Hinf*I fragments of SV40 DNA. Right: 0.5 μ g DNA of clone 3 DNA was digested with *Eco*RI, electrophoresed through a 0.7% agarose gel, blotted on to a nitrocellulose filter, hybridized with cDNA_{3'} and autoradiographed.

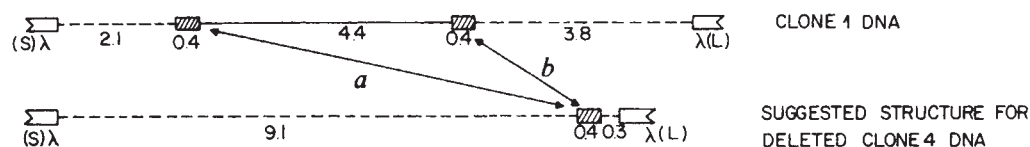
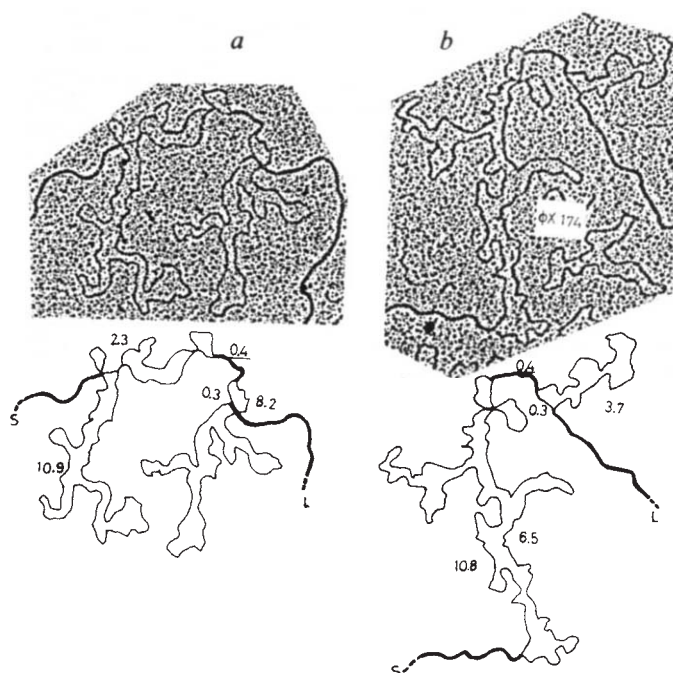


Fig. 3 Characterization of the specific deletion in cloned VL30 DNA. Top: clones 1 and 4 were previously characterized with respect to the size of VL30 DNA (solid line) and flanking DNA sequences (dotted line)¹. Boxes indicate direct terminal repeats. S and L indicate short and long arms of the λ vector, respectively. *a* and *b* indicate the two possible forms of heteroduplexes. Bottom: electron micrographs of heteroduplexes between clone 1 DNA and deleted clone 4 DNA. A representative heteroduplex of each type is shown.



propagation of cloned VL30 DNA in bacteria, also occur in the mouse genome. Lauer *et al.* have recently described deletions in human α -globin genes that are indistinguishable from deletions that occur in bacteria with respect to the location of the break-points of deletion¹¹. The availability of cloned LTR DNA sequences, devoid of other VL30 DNA sequences, provides a probe for such genomic deletions.

The biological significance of VL30 DNAs is unknown. They may represent degenerative endogenous viral genomes or may be cellular genetic elements of a proviral nature. Structurally, these genetic elements strongly resemble eukaryotic transposons^{12,13}. Shared features include similar size and reiteration frequencies, a dispersed genomic distribution, efficient transcription of a poly(A)-containing RNA and the presence of large direct repeats at the termini of these elements. Whether these DNA sequences actually transpose remains to be determined.

This work was carried out in the laboratory of Dr H. Aviv whom we thank for his help. We also thank Ms Y. Bernstein for technical assistance.

Received 20 August; accepted 20 October 1980.

A general method for site-directed mutagenesis in prokaryotes

Gary B. Ruvkun & Frederick M. Ausubel

Cellular and Developmental Biology Group, Department of Biology, Harvard University, Cambridge, Massachusetts 02138

The genetic analysis of genes from prokaryotic species for which experimental genetic systems have not yet been developed is often limited by the difficulty of producing mutations in those genes. We report here a general technique applicable to Gram-negative prokaryotes for site-directed mutagenesis of cloned DNA fragments which we have applied to the study of the symbiotic nitrogen fixation genes of *Rhizobium meliloti*. In particular, we mutagenized cloned *R. meliloti* restriction fragments in *Escherichia coli* with transposon Tn5 and then replaced the wild-type parental DNA sequences with the mutant DNA sequences in the *R. meliloti* genome. Using this method we show that an *R. meliloti* DNA restriction fragment, cloned previously on the basis of homology to *Klebsiella pneumoniae* *nif* genes¹, contains gene(s) essential for symbiotic nitrogen fixation. In addition, we use this method to construct a physical genetic map of a subset of the *R. meliloti* *nif* genes.

The bacterial species *R. meliloti* fixes nitrogen in symbiotic association with alfalfa (*Medicago sativa* L.). Establishment of symbiosis begins with the bacterial invasion of roots and culminates in the development of the root nodule, a specialized structure within which the *Rhizobium* differentiate into nitrogen-fixing bacteroids². *R. meliloti* can be grown in culture apart from its host plant, but does not normally fix nitrogen in this free-living state. Therefore, the isolation and characterization of nitrogen fixation (*nif*) mutants using standard bacterial genetic techniques would require the screening of thousands of potential bacterial mutants on individual plants. To

1. Keshet, E., Shaul, Y., Kaminchik, Y. & Aviv, H. *Cell* **20**, 431-439 (1980).
2. Hawk, R. S., Troxler, D. H., Lowy, D., Duesberg, P. H. & Scolnick, E. M. *J. Virol.* **25**, 115-123 (1978).
3. Sherwin, S. A., Rapp, U. R., Benveniste, R. E., Sen, A. & Todaro, G. J. *J. Virol.* **26**, 257-264 (1978).
4. Besmer, P., Olshevsky, A., Baltimore, D., Dolberg, D. & Fan, H. *J. Virol.* **29**, 1168-1176 (1979).
5. Scolnick, E. M., Vaas, W. C., Hawk, R. S. & Duesberg, P. H. *J. Virol.* **29**, 964-972 (1979).
6. Shimotohno, K., Mizutani, S. & Temin, H. M. *Nature* **285**, 550-554 (1980).
7. Dhar, R., McClements, W. L., Enquist, L. W. & Vande Woude, G. F. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3937-3941 (1980).
8. Sutcliffe, J. G., Shinnick, T. H., Verma, I. M. & Lerner, R. A. *Proc. Natn. Acad. Sci. U.S.A.* **77**, 3302-3306 (1980).
9. Shoemaker, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 3932-3936 (1980).
10. Majors, J. *et al. Cold Spring Harb. Symp. quant. Biol.* (in the press).
11. Lauer, J., Shen, C.-K. J. & Maniatis, T. *Cell* **20**, 119-130 (1980).
12. Cameron, J. R., Loh, E. Y. & Davis, R. W. *Cell* **16**, 739-751 (1979).
13. Finnegan, D. S., Rubin, G. M., Young, M. W. & Hogness, D. S. *Cold Spring Harb. Symp. quant. Biol.* **42**, 1053-1063 (1977).

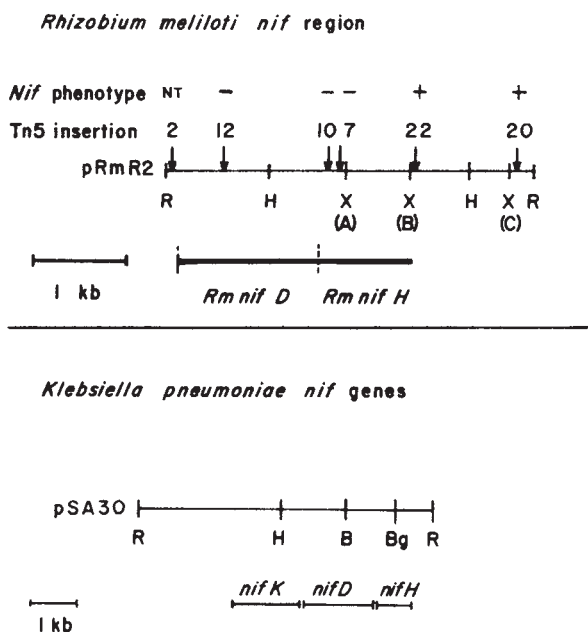


Fig. 1 Physical genetic map of *R. meliloti* *nif* genes *D* and *H*. Restriction maps of plasmid pRmR2 and each pRmR2::Tn5 plasmid were constructed using a combination of digestions in 1× universal restriction enzyme buffer (1×UREB=60 mM NaCl, 10 mM Tris pH 7.4, 6 mM MgCl₂, 6 mM β-mercaptoethanol) with restriction endonucleases *Eco*RI, *Hind*III, *Xho*I and *Bam*HI followed by gel electrophoresis in 1–1.5% agarose gels. These digested DNAs were transferred to nitrocellulose by the method of Southern⁷ and hybridized with probes derived from the *K. pneumoniae* 6.3-kilobase *Eco*RI fragment cloned in pSA30 (ref. 22). As shown in *b*, the 1.4-kilobase *Hind*III–*Bam*HI fragment of pSA30 contains the C-terminal coding region of *nifD* and the 0.7-kilobase *Bgl*II–*Eco*RI fragment contains the N-terminal coding region of *nifH* (ref. 23). These fragments were eluted from preparative agarose gels as described previously¹, and labelled with [α -³²P]TTP (350 Ci mmol⁻¹, Amersham) using the nick translation method to a specific activity of 5×10^7 c.p.m. per μg of DNA. Approximately 10⁶ c.p.m. were hybridized to the nitrocellulose sheet as described previously¹. Regions of homology in the *R. meliloti* *nif* region to *K. pneumoniae* *nifD* and *nifH* genes (shown in heavy lines) were localized by a combination of restriction endonuclease sites present in the 3.9-kilobase *Eco*RI fragment and new restriction sites generated by various Tn5 insertions. The region of homology to *K. pneumoniae* *nifD* was localized to a 1.5-kilobase region between Tn5 insertions 2 and 10. The region of homology to *K. pneumoniae* *nifH* was localized to a 1.0-kilobase region to the right of Tn5 insertion 10 and to the left of *Xho*I site B on pRmR2. R, *Eco*RI restriction site; H, *Hind*III restriction site; X, *Xho*I restriction site; B, *Bam*HI restriction site; Bg, *Bgl*III restriction site.

circumvent the tedium of this approach, we have developed new physical and genetic methods to identify and manipulate *R. meliloti* *nif* genes.

We previously showed that the *K. pneumoniae* DNA sequences coding for one subunit of nitrogenase (*nifD*) and nitrogenase reductase (*nifH*) share DNA homology with 13 widely divergent nitrogen-fixing species, including *R. meliloti*¹. We used this homology to clone a 3.9-kilobase *R. meliloti* *Eco*RI fragment in the plasmid cloning vector pACYC184 (ref. 3) (pRmR2; see Fig. 1). To obtain genetic verification that pRmR2 contains essential *nif* genes and to initiate a detailed genetic analysis of *R. meliloti* *nif* genes, we developed a method to replace the *R. meliloti* wild-type genomic *nif* genes with homologous sequences altered by transposon Tn5 mutagenesis. We chose transposon Tn5 for this mutagenesis because it confers resistance to kanamycin (Km) and neomycin (Nm) in *E. coli* and *R. meliloti*, exhibits little insertion site specificity and in general causes polar insertion mutations^{4,5}. The experimental strategy we adopted involved the series of steps summarized below and in Fig. 2.

Step I: Plasmid pRmR2 was mutagenized with Tn5 by infecting an *E. coli* strain containing pRmR2 with λ::Tn5 (ref. 6). Plasmids carrying Tn5 insertions were isolated and the locations of the Tn5 insertions determined by restriction enzyme analysis (see Fig. 2 legend). Six plasmids carrying Tn5 insertions at

different locations along the entire length of the 3.9-kilobase fragment were selected for further study (see Fig. 1). We will refer to these as 'nif region::Tn5' insertions.

Step II: A DNA transformation system has not been developed for *R. meliloti*. Therefore, to introduce the *nif* region::Tn5 insertions into *R. meliloti*, each *nif* region::Tn5 *Eco*RI fragment was recloned into the conjugative cloning vector pRK290. (Plasmid pRK290, constructed by Ditta *et al.*⁶, is a 20-kilobase derivative of the P-group conjugative plasmid RK2, confers tetracycline (Tc) resistance and contains a single *Eco*RI site suitable for cloning.) The resulting pRK290-*nif* region::Tn5 hybrid plasmids were conjugated into *R. meliloti*. Total DNA was isolated from the *R. meliloti* exconjugants and analysed by the Southern gel transfer and hybridization procedure⁷. The pRK290-*nif* region::Tn5 hybrid plasmids had the expected restriction map and replicated stably in *R. meliloti* (see Fig. 3).

Step III: Our strategy for isolating *R. meliloti* strains in which the plasmid-borne *nif* region::Tn5 fragment had replaced the genomic wild-type *nif* region was based on the assumption that homologous recombination between pRK290-*nif* region::Tn5 and the *R. meliloti* genome would occur in a small subpopulation of cells and result in the 'transfer' of the Tn5 insertion mutation from the plasmid to the genome. Cells in which this recombination event had occurred were detected by conjugation of a second P-group plasmid, pR751 (ref. 8), which is incompatible with pRK290, into the *R. meliloti* strains containing the pRK290-*nif* region::Tn5 plasmid, and simultaneous selection for neomycin resistance (retention of Tn5) and trimethoprim (Tp) resistance (conferred by pR751). All the Tp^rNm^r exconjugants were Tc^s, indicating loss of pRK290. These *R. meliloti* Tp^rNm^rTc^s exconjugants could have resulted from Tn5 transpositions to pR751 or to the *R. meliloti* genome as well as from homologous recombination between pRK290-*nif* region::Tn5 and the *R. meliloti* genome. To demonstrate that Tn5 had recombined into the *R. meliloti* genome by means of flanking *nif* DNA sequence homology, DNA was isolated from the Tp^rNm^rTc^s exconjugants, cleaved with *Bgl*II and analysed by the Southern gel transfer and hybridization technique using

Table 1 Level of nitrogenase activity in whole plants with nodules containing various *Rhizobium meliloti* strains

Strain	Ethylene produced after 24 h in acetylene gas (nmol ml ⁻¹)
Alfalfa plant alone	<1
+Rm 1021	500±100
+Rm 1021/pRK290	400±100
+Rm 1021/pR751	400±100
+Rm 1021/pRK290- <i>nif</i> region::Tn5	500±100
+Rm 1021 <i>nif</i> region::Tn5 no. 2	NT
+Rm 1021 <i>nif</i> region::Tn5 no. 12	<1
+Rm 1021 <i>nif</i> region::Tn5 no. 7	<1
+Rm 1021 <i>nif</i> region::Tn5 no. 10	<1
+Rm 1021 <i>nif</i> region::Tn5 no. 22	500±100
+Rm 1021 <i>nif</i> region::Tn5 no. 20	500±100
+Rm 1021 <i>met</i> ::Tn5	300±100

Alfalfa seeds (variety Iroquois) were sterilized for 30 min in 70% ethanol followed by 30 min in 50% commercial bleach, and then germinated in the dark in distilled water for 3 days. Individual seedlings were planted on 6-ml slants of nitrogen-free agar (0.1% CaHPO₄, 0.02% K₂HPO₄, 0.02% MgSO₄·7H₂O, 0.02% NaCl, 0.01% FeCl₃, 1.0% agar)¹³ in 18×150 mm test tubes and grown at 27°C with overhead illumination of 200 foot-candles on 16-h days for 7 days before bacterial inoculation. A freshly grown colony of *R. meliloti* grown on LB agar plus appropriate drugs was picked with a flat toothpick, suspended in 5 ml distilled water and used to inoculate five alfalfa seedlings. After 4 weeks of growth in the conditions described above, the tubes were sealed with serum stoppers, injected with 1 ml acetylene gas per tube, and incubated at room temperature in the dark for 24 h. Ethylene gas production was monitored by gas chromatography as described previously¹⁴. Control plants not inoculated with bacteria did not form nodules and no acetylene reduction was detected. All plants inoculated with *R. meliloti* formed nodules but those inoculated with strains containing *nif*::Tn5 insertions did not exhibit detectable levels of nitrogenase activity and had approximately 5–10 times the normal number of nodules per plant. Both the control plants and the plants inoculated with Nif⁻ bacterial strains were stunted and yellow after 4 weeks of growth compared with plants inoculated with Nif⁺ bacterial strains, which were green and visibly larger. NT, not tested.

32 P-labelled pRmR2 DNA as a hybridization probe. Homologous recombination of each *nif* region::Tn5 insertion from the plasmid into the *R. meliloti* genome *nif* region would result in a different predictable change in the restriction map of that region whereas transposition of Tn5 would not be expected to change the restriction map of the genomic *nif* region. Figure 3a, b, c illustrates the expected *Bgl*II restriction fragments homologous to pRmR2 for various strains intermediate in the transfer of Tn5 from pRK290-*nif* region::Tn5 to the *R. meliloti* genome. Figure 3d is an autoradiogram of an actual hybridization and shows that in the Tp^rNm^rTc^s strains examined, each *nif* region::Tn5 had replaced the normal 3.9-kilobase *nif* region in the *R. meliloti* genome through a homologous recombination event. Among 24 Tp^rNm^rTc^s colonies analysed, none contained Tn5 transpositions to new locations in the genome or to pR751, and none contained replicon fusions between pR751 and pRK290-*nif* region::Tn5 (ref. 9).

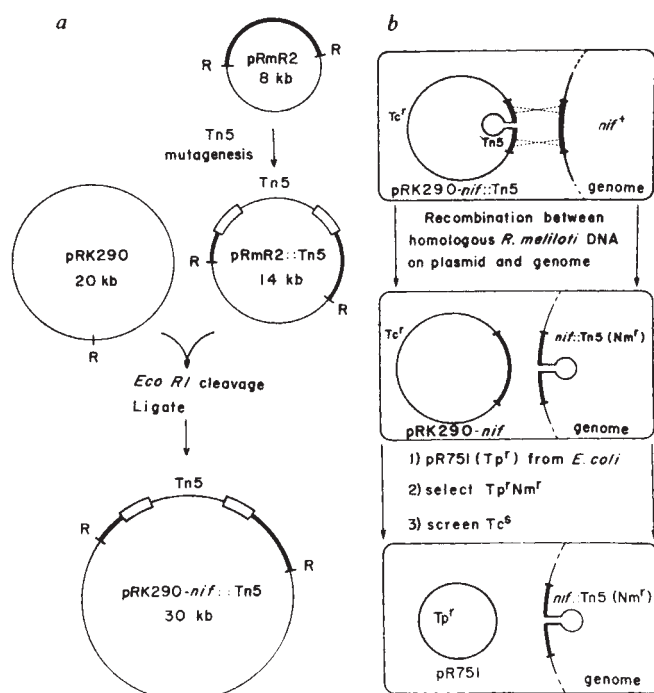
Step IV: Each *R. meliloti* strain containing a verified Tn5 insertion in the *nif* region of the genome was inoculated onto a sterile alfalfa seedling and nitrogenase activity was determined 3–4 weeks later using the acetylene reduction technique as described in Table 1 legend. Among five strains containing Tn5 insertions in the *nif* region, three had undetectable levels of nitrogenase activity (Nif⁻) on establishment of symbiosis, whereas two had normal nitrogenase activity (Nif⁺) (Fig. 1, Table 1). The construction of two Nif⁺ strains with genomic Tn5 insertions in the 3.9-kilobase *Eco*RI fragment demonstrates that the construction of the three Nif⁻::Tn5 mutations is unlikely to be an artefact of the cloning and recombinational techniques used in our gene replacement strategy. All the strains with a normal genomic *nif* region, and also plasmid pRK290-*nif* region::Tn5, were Nif⁺, indicating that none of the *nif* region::Tn5 insertions was a dominant *nif*⁻ mutation. All five strains containing Tn5 recombined into the genome were prototrophic, indicating that the Nif⁻ phenotype of three of the strains was not an indirect effect of auxotrophy.

In conjunction with the data presented in Table 1 and Fig. 3, which correlate Tn5 map position with Nif phenotype, we have used pRmR2::Tn5 plasmids to map the approximate positions of *R. meliloti* *nif* genes on the 3.9-kilobase *Eco*RI fragment. In these experiments we used specific *K. pneumoniae* restriction fragments carrying regions of genes *nifD* (nitrogenase) and *nifH* (nitrogenase reductase) as hybridization probes to various restriction digests of pRmR2 and pRmR2::Tn5 plasmids (Fig. 1). We found that sequences within both *K. pneumoniae* *nifD* and *nifH* are conserved in *R. meliloti*. The conserved *R. meliloti* '*nifD*' sequences are localized to 1.5 kilobases between Tn5 insertions 2 and 10, and the conserved *R. meliloti* '*nifH*' sequences are localized to 1.0 kilobase between Tn5 insertion 10 and *Xho*I site B indicated in Fig. 1.

As expected on the basis of the interspecies DNA homology with *K. pneumoniae* *nif* genes, those Tn5 insertions which cause a Nif⁻ phenotype in *R. meliloti* are localized near or in these regions of homology, whereas the two Tn5 insertions which have no effect on *nif* gene expression are located at the other end of the 3.9-kilobase *R. meliloti* *Eco*RI fragment and not in the region of interspecies *nif* gene homology. Because Tn5 insertion 22 does not cause a Nif⁻ phenotype, the *nif* gene *H* equivalent in *R. meliloti* probably starts to the left of this Tn5 insertion (Fig. 1). The protein subunits for nitrogenase and nitrogenase reductase have average molecular weights of 55,000 and 28,000, respectively, in other *Rhizobium* species^{10,11}; therefore, the minimum length of DNA necessary to code for these proteins should be 2.2 kilobases. Thus, it is likely that the 2.6 kilobases between Tn5 insertion 22 and Tn5 insertion 2 contains all of *nifH* and at least part of *nifD*.

An important conclusion to be drawn from the present data is that cloned *R. meliloti* DNA sequences homologous to *K. pneumoniae* *nifD* and *nifH* are essential for symbiotic nitrogen fixation. The data rule out the possibility that the DNA homology between *K. pneumoniae* *nif* genes and *R. meliloti* DNA is due to cryptic *nif* genes not functional in symbiotic

Fig. 2 Outline of experimental strategy used to introduce specific Tn5 mutations into the *R. meliloti* genome. **a**, Mutagenesis of pRmR2 with Tn5 and recloning of *nif* region::Tn5 fragments into pRK290. *E. coli* strain HB101/pRmR2 (*thr* *leu* *thi* *recA* *hsdR* *hsdM* *pro* *Str*^r *Tc*^s) was grown at 37 °C in LB medium containing 0.2% maltose and 10 µg ml⁻¹ Tc to a density of 5 × 10⁸ cells per ml and infected with λ221 *rex*::Tn5 *cl*857 *O*_{am}^s *P*_{am}²⁹ (ref. 5) at a multiplicity of 0.1 particles per cell. Approximately 5 × 10³ kanamycin-resistant survivors were obtained per plate when 0.2 ml of infected cells were plated on LB agar containing 20 µg ml⁻¹ Km at 32 °C. Colonies from three plates were resuspended in 10 ml of 10 mM MgSO₄, plasmid DNA was isolated¹⁵ and used to transform CaCl₂-treated strain HB101 (ref. 16), and Tc^r and Tc^rKm^r transformants were selected on LB agar containing 10 µg ml⁻¹ Tc and 20 µg ml⁻¹ Km. Approximately 20 Tc^rKm^r transformants were obtained per 10⁴ Tc^r transformants. Plasmid DNA was prepared from 26 Tc^rKm^r colonies using a small-scale purification procedure¹⁷, and the plasmid DNA was digested in UREB with *Eco*RI. Because Tn5 is 5.7 kilobases and does not contain an *Eco*RI site, insertions of Tn5 into the 3.9-kilobase *nif* homologous *R. meliloti* *Eco*RI restriction fragment were monitored (using agarose-gel electrophoresis) by the appearance of a 9.6-kilobase *Eco*RI restriction fragment and disappearance of the 3.9-kilobase *Eco*RI restriction fragment. Among the 26 plasmids, 11 contained Tn5 insertions in the 3.9-kilobase *Eco*RI fragment and the sites of these Tn5 insertions along the 3.9-kilobase *Eco*RI restriction fragment were determined by double restriction endonuclease digestions of each pRmR2::Tn5 plasmid in 1X UREB with *Xho*I and *Eco*RI or *Hind*III and *Eco*RI. Six pRmR2::Tn5 plasmids with Tn5 insertions distributed along the entire length of the 3.9-kilobase *Eco*RI fragment were chosen for further analysis. A 0.5-µg aliquot of each of the six pRmR2::Tn5 plasmids was digested with *Eco*RI as described above and mixed with a 0.5-µg aliquot of pRK290 DNA which had also been digested with *Eco*RI. The mixed DNAs were ligated with 1 unit of T4 DNA ligase, as described elsewhere¹⁴, and the ligated DNA was used to transform *E. coli* strain MM294 (*end* *hsdR* *pro*). Following a nonselective growth in 1 ml LB for 2 h at 32 °C, a 0.1-ml aliquot was diluted into 5 ml LB containing 10 µg ml⁻¹ Tc and 20 µg ml⁻¹ Km and grown to saturation at 32 °C. 0.1 ml of this culture was mated simultaneously with 0.1 ml *R. meliloti* strain Rm1021 (*nif*⁺ *Str*^r) and 0.1 ml MM294/pRK2013, and incubated on an LB agar plate overnight at 32 °C. (Plasmid pRK2013 (ref. 18) contains the *tra* genes of RK2 cloned in plasmid ColE1 and complements pRK290 *in trans* for conjugation of pRK290.) *Str*^rTc^rNm^r *R. meliloti* exconjugants were selected on LB agar containing 250 µg ml⁻¹ streptomycin (*Str*), 5 µg ml⁻¹ Tc and 20 µg ml⁻¹ Nm. Because pRK2013 (which confers Nm^r) cannot replicate in *R. meliloti*⁶, this selection yielded *R. meliloti* exconjugants containing pRK290-*nif* region::Tn5 plasmids which arose at a frequency of 10⁻³. **b**, Selection of *R. meliloti* strains in which the *nif* region::Tn5 mutations had recombined in the genome. *E. coli* strain J53/pR751 (ref. 8) was conjugated with each of the *R. meliloti* M1021/pRK290-*nif* region::Tn5 strains as described above and *Str*^rTp^rNm^r exconjugants were selected on LB agar containing 250 µg ml⁻¹ *Str*, 700 µg ml⁻¹ Tp and 30 µg ml⁻¹ Nm. Exconjugants arose at a frequency of 10⁻⁶ and 24 of these (four from each of the six pRK290-*nif* region::Tn5 strains) were analysed as described in Fig. 3 legend. *R. meliloti* *nif* region DNA is shown in heavy lines; Tn5 inverted repeat DNA shown as open boxes (R = *Eco*RI restriction site).



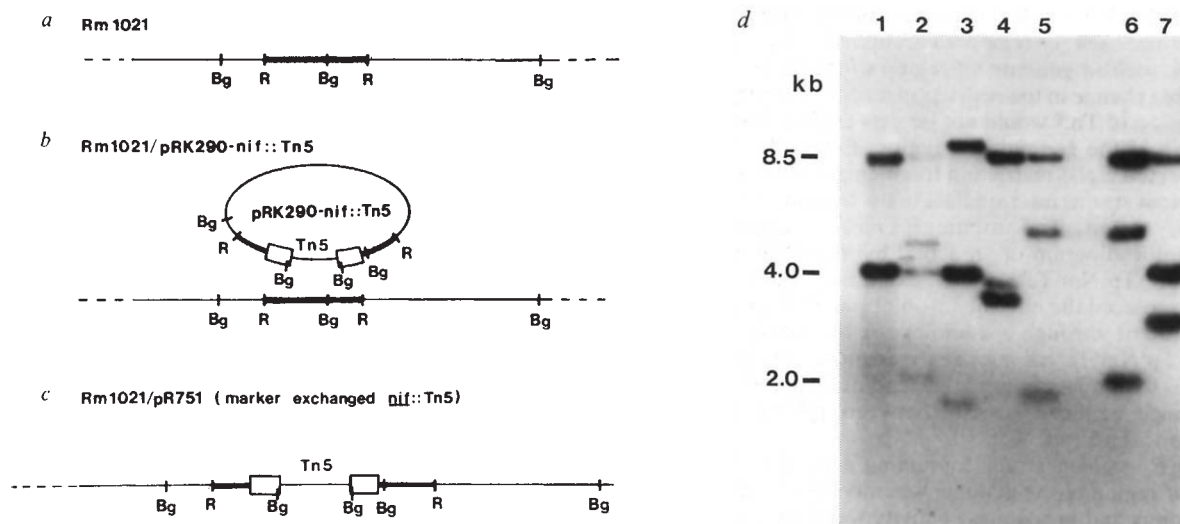


Fig. 3 Change in the *Rhizobium meliloti* *nif* region restriction map due to homologous recombination of *nif* region::Tn5 from pRK290 to the *R. meliloti* genome. A restriction map of the *nif* region in the *R. meliloti* genome was constructed as follows. Total DNA was isolated from *R. meliloti* strain Rm1021, using the Marmur technique¹⁹; approximately 1 µg of DNA was digested with restriction endonuclease *Bgl*II or *Eco*RI (5 U Bethesda Research Laboratories) or both, and restriction fragments were separated on 0.7% agarose gels and transferred to nitrocellulose by the method of Southern⁷ as described elsewhere². Plasmid pRmR2 was isolated by the cleared lysate technique¹⁵ and labelled by nick translation^{20,21} with [α -³²P]TTP (350 Ci mmol⁻¹, Amersham) and DNA polymerase I (Boehringer Mannheim) to a specific activity of 5×10^7 c.p.m. per µg of DNA, and 10^6 c.p.m. of labelled DNA probe was hybridized to each 10×15 -cm nitrocellulose filter and exposed to Kodak XR-5 film as described elsewhere². **a**, Restriction map of the normal *nif* region of *R. meliloti*. Hybridization of pRmR2 probe to *Bgl*II-digested Rm1021 DNA as described above should yield hybridization bands at 8.5 and 4.0 kilobases which overlap the 3.9-kilobase *Eco*RI fragment (shown in heavy lines) cloned in pRmR2. (The cloning vector pACYC184 does not hybridize to *R. meliloti* DNA; unpublished results.) **b**, Restriction map of the two regions of homology to pRmR2 in Rm1021 strains containing plasmid pRK290-*nif* region::Tn5. Tn5 insertion into any *Bgl*II restriction fragment adds two *Bgl*II sites each located 1.5 kilobases from the Tn5 insertion site. Hybridization of pRmR2 probe to *Bgl*II-digested DNA from a Rm1021/pRK290-*nif* region::Tn5 strain as described above should yield the normal genomic 8.5- and 4.0-kilobase hybridization bands plus three additional hybridization bands due to the four pRK290-*nif* region::Tn5 plasmid. **c**, Restriction map of the *nif* region of *R. meliloti* following homologous recombination of Tn5 into the parent 3.9-kilobase *Eco*RI restriction fragment. Hybridization of ³²P-labelled pRmR2 DNA to *Bgl*II-digested DNA from a strain containing a genomic Tn5 insertion into the parental *Eco*RI fragment cloned in pRmR2 should yield three bands, one of which should be either 4.0 or 8.5 kilobases (same as wild type) and two which should vary in size. The sum of the lengths of these latter two bands should be either 8.5 kilobases or 4.0 plus 3.0 kilobases (2×1.5 kilobases of Tn5 DNA). Because each Tn5 insertion which was used in the marker-exchange procedure was previously mapped on the parent pRmR2::Tn5 plasmid, a unique *Bgl*II restriction hybridization map for the *nif* region could be predicted for each genomic Tn5 insertion. For example, Tn5 insertion 12 is located to the 'left' of the *Bgl*II site on pRmR2 as drawn here. As predicted, hybridization of ³²P-labelled pRmR2 DNA to *Bgl*II-digested DNA from a strain containing Tn5 insertion 12 recombined into the *R. meliloti* genome yielded hybridization bands of 8.5, 3.8 and 3.4 kilobases (**d**, lane 4). Each of the other insertion mutations also gave the predicted hybridization patterns (**d**). **d**, Autoradiogram of ³²P-labelled pRmR2 hybridized to DNA from various *R. meliloti* strains (1 µg), digested with *Bgl*II (5 U, in 1X UREB), separated by 1.0% agarose-gel electrophoresis and transferred to nitrocellulose as described above from the following strains: lane 1: Rm1021/pR751 (hybridization bands at 8.5 and 4.0 kilobases); lane 2: Rm1021/pRK290-*nif* region::Tn5 no. 10 (hybridization bands at 20, 8.5, 4.8, 4.0 and 2.0 kilobases); lane 3: Rm1021 *nif* region::Tn5 no. 22/pR751 (hybridization bands at 9.8, 4.0 and 1.7 kilobases); lane 4: Rm1021 *nif* region::Tn5 no. 12/pR751 (hybridization bands at 8.5, 3.7 and 3.3 kilobases); lane 5: Rm1021 *nif* region::Tn5 no. 7/pR751 (hybridization bands at 8.5, 5.2 and 1.8 kilobases); lane 6: Rm1021 *nif* region::Tn5 no. 10/pR751 (hybridization bands at 8.5, 5.1 and 1.9 kilobases); lane 7: Rm1021 *nif* region::Tn5 no. 20/pR751 (hybridization bands at 8.5, 4.0 and 2.9 kilobases). *Bgl*II-digested DNA from strains M1021/pRK290, M1021/pR751 and M1021 all hybridized equivalently to pRmR2 probe (data not shown). The lower portion of the autoradiograph was photographically enhanced to show the faint smaller hybridization bands. *Bgl*, *Bgl*II restriction site; *R*, *Eco*RI restriction site; kb, kilobases.

nitrogen fixation or due to homology with genes evolutionarily (or fortuitously) related to *K. pneumoniae* *nif* genes. Based on the interspecies homology with *K. pneumoniae* *nifD* and *nifH* and on the mutational analysis described here, we conclude that *R. meliloti* contains genes equivalent to *K. pneumoniae* *nifD* and *nifH*, although we cannot yet assign these homologous *R. meliloti* *nif* genes to separate complementation groups. Because we expect other nitrogen-fixing species to have similar genes, we propose the following nomenclature for nitrogenase genes from various organisms. Using the letters already assigned to *nif* genes in *K. pneumoniae*, we suggest adding the first letters of the genus and species names. Thus *K. pneumoniae* *nifH* would be written *Kp nifH* and the corresponding gene in *R. meliloti* would be written *Rm nifH*. This nomenclature is analogous to that already in use for distinguishing nitrogenase proteins isolated from different species¹².

Because the techniques described use a wide host-range conjugative cloning vector and do not necessarily require previous identification of genetic function of the cloned DNA, they should be applicable to fine-structure genetic analysis of a wide variety of Gram-negative prokaryotes. These techniques should also be useful in the initiation of genetic studies of DNA fragments cloned from Gram-negative prokaryotes by non-genetic criteria, for example, homology to inducible RNA or homology to specific DNA sequences. Finally, DNA fragments identified and cloned on the basis of transposon insertional inactivation can be further mutagenized using these techniques.

We thank G. Ditta for providing plasmids pRK290 and pRK2013 before publication, G. Jacoby for providing plasmid

pR751, F. Lang for advice on decreasing background in Tp selections, and R. Hyde for preparation of this manuscript. This work was supported by USDA/SEA/CRGO grant 7900481 and NSF grant PCM7806834 awarded to F. M. A.

Received 21 July; accepted 16 October 1980.

1. Ruvkun, G. B. & Ausubel, F. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 191-195 (1980).
2. Vincent, J. M. in *The Biology of Nitrogen Fixation* (ed. Quispel, A.) 265-341 (North-Holland, Amsterdam, 1974).
3. Chang, A. C. Y. & Cohen, S. N. *J. Bact.* **134**, 1141-1156 (1978).
4. Jorgenson, R. A., Rothstein, S. J. & Reznikoff, W. S. *Molec. gen. Genet.* **177**, 65-72 (1979).
5. Berg, D. E. in *DNA Insertion Elements, Plasmids and Episomes* (eds Bukhari, A. I., Shapiro, T. A. & Adhya, S. L.) 205-212 (Cold Spring Harbor Laboratory, New York, 1977).
6. Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. *Proc. natn. Acad. Sci. U.S.A.* (submitted).
7. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
8. Jobanputra, R. S. & Datta, N. *J. med. Microbiol.* **7**, 169-177 (1974).
9. Kleckner, N. *Cell* **11**, 11-23 (1977).
10. Isreal, D. W., Howard, R. L., Evans, H. J. & Russell, S. A. *J. biol. Chem.* **249**, 500-508 (1974).
11. Whiting, M. J. & Dilworth, M. J. *Biochim. biophys. Acta* **371**, 337-351 (1974).
12. Burns, R. C. & Hardy, R. W. F. *Nitrogen Fixation in Bacteria and Higher Plants*, 64 (Springer, New York, 1975).
13. Vincent, J. M. in *A Manual for the Practical Study of Root-Nodule Bacteria*, 75 (Blackwell, Oxford, 1970).
14. Cannon, F. C., Riedel, G. E. & Ausubel, F. M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2963-2967 (1977).
15. Clewell, D. B. & Helinski, D. R. *Proc. natn. Acad. Sci. U.S.A.* **62**, 1159-1166 (1969).
16. Cohen, S. N., Chang, A. C. Y. & Hsu, C. L. *Proc. natn. Acad. Sci. U.S.A.* **69**, 2110-2124 (1972).
17. Klein, R. D., Selsing, E. & Wells, R. D. *Plasmid* **3**, 88-91 (1980).
18. Figurski, D. H. & Helinski, D. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1648-1652 (1979).
19. Marmur, J. *J. molec. Biol.* **3**, 208-218 (1961).
20. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237-251 (1977).
21. Maniatis, T., Jeffrey, A. & Kleid, D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1184-1188 (1975).
22. Cannon, F. C., Riedel, G. E. & Ausubel, F. M. *Molec. gen. Genet.* **174**, 59-66 (1979).
23. Riedel, G. E., Ausubel, F. M. & Cannon, F. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2866-2870 (1979).

Escherichia coli ribosomal protein S8 feedback regulates part of *spc* operon

Dennis Dean, John L. Yates & Masayasu Nomura*

Institute for Enzyme Research, Departments of Genetics and Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706

In *Escherichia coli* the genes coding for the 52 ribosomal proteins (r-proteins) are organized into a number of transcription units located at various regions on the bacterial genome. The expression of r-protein genes is balanced so that individual r-protein synthesis rates change coordinately in response to changing environmental conditions, and significant amounts of free r-proteins do not exist in the cellular pool. We have suggested a model for the balanced regulation of r-protein gene expression, namely that r-protein synthesis and ribosome assembly are coupled so that r-proteins not incorporated into ribosomes prevent the further translation of r-protein mRNA by a feedback regulatory mechanism¹. The model was tested *in vitro* by examining the effect of purified r-proteins on DNA directed r-protein synthesis^{2,3}, and *in vivo* by examining the effect of overproduction of certain r-proteins on the synthesis rates of other r-proteins^{4,5}. *In vitro* experiments have revealed that some r-proteins (L1, L4, L10, S4 and S8) can selectively inhibit the synthesis of r-proteins whose genes are in the same operon as their own, and that this specific feedback regulation occurs at the level of translation rather than at the level of transcription of mRNA^{2,3,6,7}. Regulatory roles for L1, S4 and L4 have also been established by *in vivo* experiments^{4,5}. We have studied further the feedback regulatory properties of S8 *in vivo* and *in vitro*, and report here that the protein regulates a part of the *spc* operon.

Figure 1 shows the *in vitro* effects of purified S8 addition on synthesis of r-proteins encoded in the *spc* operon. Protein synthesis was directed by a DNA fragment containing most genes of the *spc* operon (L14, L24, L5, S14, S8, L6 and L18) or by λ spc1 DNA. λ spc1 transducing phage carries the genes in the *spc* and α operons (see Fig. 2). Radioactive proteins were precipitated from reaction mixtures with specific antisera and analysed by SDS-gel electrophoresis followed by autoradiography. Addition of S8 (2.9 μ M) results in specific inhibition of L5 and S14 synthesis (Fig. 1a, 5–8), but only slight or no inhibition of other proteins from the *spc* operon. Densitometric tracings of films from several such experiments showed that S8 inhibits the synthesis of L5 and S14 by 75–90% but inhibits the synthesis of L14, L24, S8, L6, L18 and S5 by less than 30%. The effect of S8 addition on the synthesis of L30 and L15 was not examined.

We have previously reported that L24 synthesis was also inhibited by S8 (ref. 2). However, we have now established that S8 has no significant inhibitory effect on L24 synthesis in the DNA-directed system using immunoprecipitation methods and consider that the inhibition originally observed was inhibition of synthesis of S14, which co-migrates with L24 in the SDS gels used. As we have previously shown², addition of other r-proteins such as L1 or S4 has no inhibitory effects on synthesis of r-proteins contained within the *spc* operon. It is significant that expression from the promoter proximal genes (L14 and L24) is not inhibited by S8. These results support our previous conclusion that regulatory r-proteins act at the level of translation and not at the level of transcription originating from the promoter.

To test whether the regulatory effects of S8 observed *in vitro* also reflect *in vivo* regulatory properties of S8, we examined the

effect of S8 over-production on synthesis rates of other r-proteins *in vivo*. A DNA fragment containing three genes from the *spc* operon (S8, L6 and L18) was cloned into a plasmid vector containing the *lac* operator and promoter so that the synthesis of S8, L6 and L18 were then controlled by the *lac* regulatory elements (pNO1017; see Fig. 2). Another plasmid (pNO1018) that contains only the S8 gene fused to the *lac* operator–promoter was derived from pNO1017 (see Fig. 2).

Effects of over-production of S8, L6 and L18, or S8 alone on the synthesis rates of other individual r-proteins were examined by stimulating transcription from *lacZp* in strains which harbour the recombinant plasmids carrying r-protein genes fused to *lacZp*. Exponentially growing cells were treated with an inducer of the *lac* operon, 1 mM isopropyl thiogalactoside (IPTG), to stimulate synthesis of the various cloned r-protein genes. Synthesis rates of individual r-proteins were then analysed by labelling cells with [³H]lysine for 1 min followed by a 1-min chase with nonradioactive lysine. Relative synthesis rates were obtained by comparing the synthesis rates of individual r-proteins determined after IPTG induction with the synthesis rates of individual r-proteins before IPTG addition.

Table 1 Relative synthesis rates of ribosomal proteins after induction by IPTG

r-protein	Strains	
	NO2320 (carries pNO1017)	NO2321 (carries pNO1018)
S4	1.21	1.14
S5	0.50*	0.25*
S7	1.07	1.10
S8	4.10*	2.25*
S13	1.13	1.12
S14	0.37*	0.37*
S20	1.21	1.13
L1	1.00	1.00
L2	1.13	1.13
L3	1.00	1.00
L5	0.36*	0.36*
L6	3.50*	0.40*
L13	0.99	0.97
L14	1.14	1.02
L15	0.47*	0.57*
L16	1.02	1.05
L17	1.02	1.13
L18	3.55*	0.31*
L19	1.10	0.99
L22	1.24	1.22
L24	0.99	1.04
L28	1.23	1.04
L30	0.26*	0.30*

For labelling experiments cells were grown in a synthetic minimal medium¹² supplemented with 0.4% glycerol, all amino acids (except methionine and lysine) at 50 μ g ml⁻¹, and thiamine at 2 μ g ml⁻¹. Cells were grown at 37°C to 2×10^8 ml⁻¹. Samples of cells were pulse-labelled with [³H]-lysine (646 pmol ml⁻¹; 80.5 Ci mmol⁻¹) for 1 min followed by a 1 min 'chase' with excess nonradioactive lysine. Cells were labelled before (control cells), and 10 min after addition of IPTG to 1 mM (experimental cells). Cells were rapidly chilled and then mixed with a suitable amount of [¹⁴C]-lysine-labelled carrier cells. [¹⁴C]-labelled cells were prepared by growing cells in the presence of [¹⁴C]-lysine for several generations. r-Proteins were extracted and separated by two-dimensional gel electrophoresis¹³. After the gels had been stained, the spots corresponding to the indicated r-proteins were extracted, the gels were oxidized with a Packard sample oxidizer, and ³H and ¹⁴C measured separately. The ³H/¹⁴C ratio in each protein in the experimental cells was then compared with that in control cells using the expression:

$$\frac{[^3\text{H}/^{14}\text{C}] \text{ in protein } i \text{ in experimental cells}}{[^3\text{H}/^{14}\text{C}] \text{ in protein } i \text{ in control cells}}$$

The ratios obtained were then normalized to the ratio obtained for L1 (indicated by a box). Values given in the table are the average of two independent experiments. *Values significantly different from 1.0.

* To whom correspondence should be addressed.

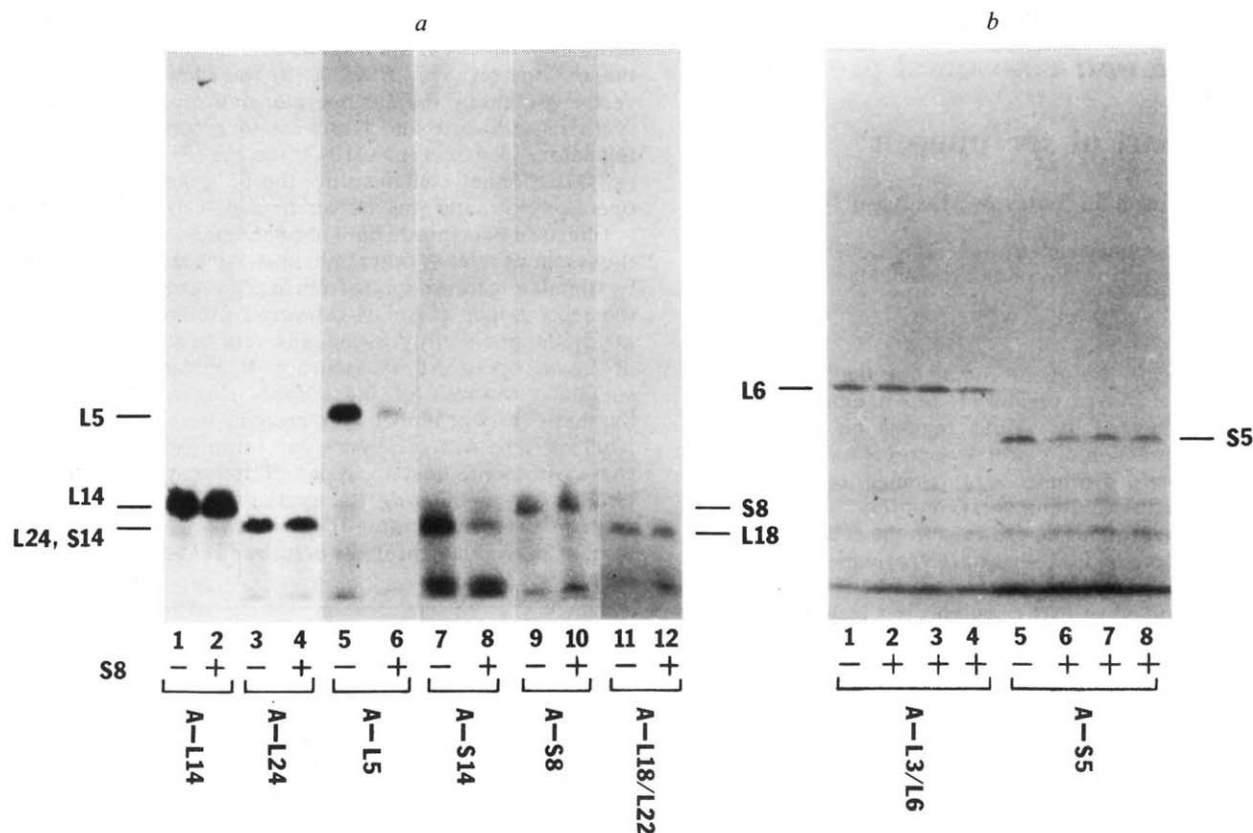
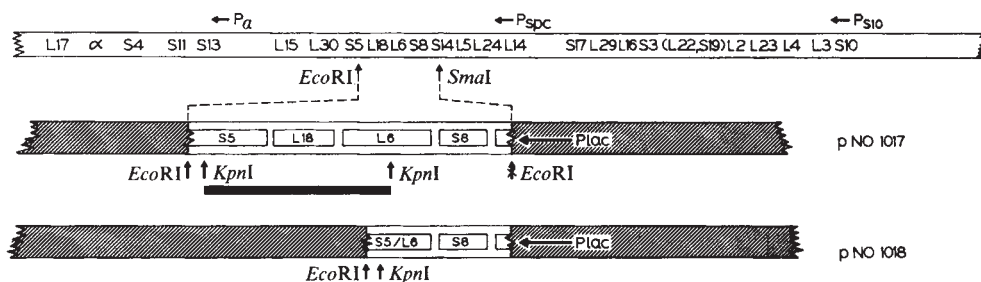


Fig. 1 Effects of S8 on protein synthesis from the *spc* operon *in vitro*. *a*, *In vitro* protein synthesis was carried out as described elsewhere² using the 10% *Eco*RI DNA fragment of λ spc2 transducing phage as template. This fragment carries genes between an *Eco*RI site in the S3 gene from the S10 operon and the *Eco*RI site in the S5 gene from the *spc* operon (see Fig. 2). Protein was labelled using ³⁵S-methionine (160 mCi per μ mol, 15 μ M). Two reaction mixtures (80 μ l) received either no S8 (control sample) or 3 μ g S8. The reactions were terminated after 60 min at 37 °C, then 3 μ g S8 was added to the control sample. 10- μ l aliquots (5 μ l for 1 and 2) were taken from reaction mixtures which had been incubated in the absence or presence of S8, as indicated, and radioactive proteins synthesized were precipitated as described previously² by treating with antisera to specific r-proteins. The antiserum used for a particular experiment is shown at the bottom of the figure (A followed by the r-proteins used to raise that antiserum). A-L18/L22 is an antiserum raised against a mixture of r-proteins L18 and L22. Polyacrylamide gel electrophoresis was carried out according to the procedure of Laemmli⁸ in 13.5% acrylamide-SDS gels. Gels were impregnated with diphenyloxazole (PPO)⁹ and exposed to pre-flashed X-ray film¹⁰. In the autoradiograms shown the direction of migration is from the top to the bottom. The origin of radioactive protein bands migrating to the bottom of the gel is not known. No consistent pattern in the amounts of this material was observed in relation to the presence or absence of S8 in the reaction mixture. *b*, Proteins were synthesized using λ spc1 DNA as template and labelled with ³H-lysine (80 mCi per μ mol, 15 μ M). Analysis was done as described above using 80 μ l reaction mixtures with no S8 added during incubation (lanes 1, 5), 1.8 μ g S8 added (lanes 2, 6), 2.7 μ g S8 (lanes 3, 7) and 3.6 μ g S8 (lanes 4, 8). To analyse L6, an antiserum against a mixture of L3 and L6 (A-L3/L6) was used.

Fig. 2 Construction of hybrid plasmids pNO1017 and pNO1018. The plasmid vector was pBGP120 (ref. 11), which is a derivative of RSF2124 and contains a region of the *Aplac* genome including the *lac* promoter and the main part of the *lacZ* gene. The approximate position of the *lacZp* ('Plac' in the figure) and the direction of transcription from *lacZp* (the thick horizontal arrow) are indicated. The vector region is hatched and the bacterial region is open. Plasmid pNO1017 was constructed by ligating *Eco*RI-digested pBGP120 and the *Eco*RI-SmaI fragment indicated in the figure purified from λ spc-DNA²: the fusion of the *Sma*I-*Eco*RI ends resulted in loss of the *Eco*RI and *Sma*I sites (indicated by a cross through an *Eco*RI site in pNO1017). pNO1018 was constructed by digesting pNO1017 with *Kpn*I and ligation to delete the region indicated by the cross-hatched region. The positions of the *Kpn*I sites are approximate.



Over-production of r-proteins S8, L6 and L18 resulted in an inhibition of synthesis of certain other r-proteins (L5, S14, S5, L30 and L15) contained within the *spc* operon (Table 1). Synthesis rates for the promoter proximal r-proteins (L14 and L24), however, were not affected. Similarly over-production of S8 alone inhibited the synthesis of L5, S14, L6, L18, S5, L30 and L15, but not the synthesis of L14 or L24. No significant changes in the synthesis rates of other r-proteins not contained in the *spc* operon were observed as a consequence of S8, L6 or L18 over-production. The *in vivo* results, therefore, confirm our *in vitro* results—S8 is capable of inhibiting synthesis of certain

r-proteins encoded in the *spc* operon. Similar to previous results obtained with the S10 and α operons, synthesis of proteins from promoter distal genes were inhibited *in vivo* but not *in vitro*. As discussed previously^{3,5}, we believe that the observed regulatory effects on promoter distal genes observed *in vivo* are the consequence of a polar effect caused by direct inhibition of translation of upstream mRNA. The *in vivo* polar effect could be due to premature transcription termination, increased degradation of distal mRNA, or translation of distal cistrons requiring ribosomes that initiated in an earlier cistron. The important observation in the present study is that, unlike

the previously studied r-protein operons, inhibition occurs at the third gene in the operon rather than the first. This result clearly indicates that inhibition of synthesis of specific r-proteins contained in the *spc* operon by S8 cannot occur at the level of initiation of transcription from the *spc* promoter. This conclusion is consistent with our previous *in vitro* observations that autogenous feedback regulation by repressor r-proteins occurs at the translation step rather than the transcription step^{2,3}. It is particularly intriguing that synthesis of r-proteins that are coordinately regulated and whose genes are contained within a single transcription unit are apparently regulated independently at the translational level. The method of regulation of synthesis of r-proteins L14 and L24 is unknown.

We thank Kristin Ellingsen for technical help. This work was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, by grant GM-20427 from the NIH, and by grant PCM 79-10616 from the NSF. D.D. is supported by NIH postdoctoral fellowship grant GM-07553.

Received 29 August; accepted 29 October 1980.

1. Fallon, A. M., Jinks, C. S., Strycharz, G. D. & Nomura, M. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3411-3415 (1979).
2. Yates, J. L., Arfsten, A. E. & Nomura, M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1831-1841 (1980).
3. Yates, J. L. & Nomura, M. *Cell* **21**, 517-522 (1980).
4. Zengel, J. M., Mueckl, D. & Lindahl, L. *Cell* **21**, 523-535 (1980).
5. Dean, D. & Nomura, M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3590-3594 (1980).
6. Brot, N., Caldwell, P. & Weissbach, H. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2592-2595 (1980).
7. Fukuda, R. *Molec. gen. Genet.* **178**, 483-486 (1980).
8. Laemmli, U. K. *Nature* **227**, 680-685 (1970).
9. Bonner, W. M. & Laskey, R. A. *Eur. J. Biochem.* **46**, 83-88 (1974).
10. Laskey, R. A. & Mills, A. D. *Eur. J. Biochem.* **56**, 335-341 (1975).
11. Polisky, B., Bishop, R. J. & Gelfand, D. H. *Proc. natn. Acad. Sci. U.S.A.* **73**, 3900-3904 (1976).
12. Clark, D. J. & Maaløe, O. J. *J. molec. Biol.* **23**, 99-112 (1967).
13. Howard, G. A. & Traut, R. R. *FEBS Lett.* **29**, 177-180 (1973).

Oriented fibrin gels formed by polymerization in strong magnetic fields

J. Torbet

Max-Planck-Institut für Festkörperforschung, Hochfeld-Magnetlabor 166X, F-38042 Grenoble Cedex, France

J. -M. Freyssinet & G. Hudry-Clergeon

Laboratoire d'Hématologie, DRF, FRA 21 INSERM, Centre d'Etudes Nucléaires de Grenoble, 85 X, F-38041 Grenoble Cedex, France

Fibrinogen is a soluble plasma protein which, after cleavage by the specific proteolytic enzyme thrombin, polymerizes to form the filamentous fibrin network during blood clotting (see refs 1 and 2 for reviews). Fibrinogen has a molecular weight of 340,000 and is composed of two identical halves, each containing three peptide chains designated A α , B β and γ . Fibrin monomers are produced by thrombin which releases the small negatively charged fibrinopeptides A and B. The overall shape of the fibrinogen molecule has not been unequivocally established^{1,2}. The trinodular, elongated (~450 Å long) structure proposed by Hall and Slayter³ is the most widely accepted model and it has obtained additional support from recent work⁴⁻⁶. Fibrin monomers are also about 450 Å long⁷ and in fibres they probably have a half-staggered arrangement along the axis^{7,8}. The fibres are an assembly of protofibrils whose structure and packing are not reliably known. We report here that highly oriented fibrin gels are formed when polymerization takes place slowly in a strong magnetic field. It is shown that the protofibrils pack into a three-dimensional crystalline lattice. We introduce magnetically induced birefringence as a potential tool for studying polymerization and briefly speculate on the applications of strong magnetic fields.

The sigmoidal variation of the birefringence (Fig. 1), observed when a fibrin gel is formed in a strong magnetic field, can be qualitatively explained as follows. Near the beginning there is little induced birefringence because the fibrinogen molecules and fibrin monomers present have only a small diamagnetic anisotropy⁹. The magnetic orienting energy is much less than the thermal energy kT and consequently they orient poorly. The upsurge in the birefringence commences with polymerization. Now many monomers come together in an ordered way, the diamagnetic anisotropy of the aggregates is many times that of a monomer and the magnetic orienting energy is sufficiently large to allow a high degree of orientation. The value of $\Delta n/C$, where n is the refractive index and C the concentration, at the end of the reaction increases as the rate of polymerization is reduced until a limiting value is reached which corresponds to the maximum alignment of the fibres in the field used. Thus, when polymerization proceeds too rapidly, the interlinked network develops before the nascent fibres have had time to orient themselves fully.

Scanning micrographs of unoriented and magnetically oriented gels are compared in Fig. 2. The samples suffered distortion while being dried in preparation for electron microscopy. Nevertheless, the orientation of the gel can be easily seen to parallel the field direction.

Figure 3 shows the low-angle neutron diffraction pattern obtained from magnetically oriented fibrin gels. In addition to the familiar¹⁰⁻¹² 223 Å (± 5 Å)-spaced meridional reflections, there are equatorial and other Bragg reflections which indicate that the fibrin monomers are arranged in a regular three-dimensional lattice. The sharpness of the meridional reflections, which is due to a fibre axial periodicity, shows that there is a high degree of crystallinity along the fibre axis¹⁰. These reflections exhibit little arcing and when the gel is tilted slightly from the vertical, the intensity of the higher orders is markedly reduced. Hence, the fibres are highly oriented along the magnetic-field direction. Rotating the gel about the vertical axis does not alter the non-meridional reflections, showing that the samples are rotationally symmetrical and therefore there can be little long-range rotational order between fibres.

The equatorial diffraction arises from the regular, lateral arrangement of the fibrin molecules. In the pattern there is a discrete peak at 184 ± 5 Å and an extended band with a maximum at about $184/5^{1/2}$ Å (Figs 3, 4). The diffraction is not detailed enough to deduce directly the parameters of the unit cell perpendicular to the fibre axis. However, from simple

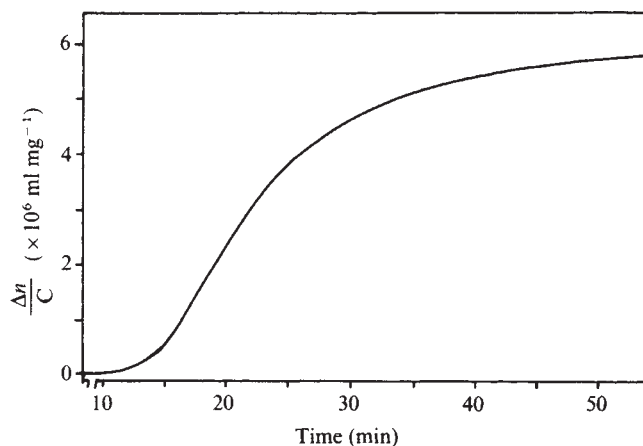


Fig. 1 The change with time of the birefringence per unit concentration of protein $\Delta n/C$ (C in mg ml^{-1}) when polymerization is performed in a constant magnetic field of 11 T ($1 \text{ T} = 10^4 \text{ G}$). The birefringence was measured at $6,328 \text{ Å}$ by means of a mechanically driven Babinet Soleil compensator. A small volume of thrombin (0.10 NIH units) was used to induce polymerization at 22°C of a 1-ml solution of fibrinogen (4.8 mg ml^{-1} in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.10 M NaCl and 0.5 mM EDTA).

packing considerations one expects the basal unit cell, perpendicular to the fibre axis, to be nearly square or hexagonal. The position of the maximum at $184/5^{1/2}$ Å (Fig. 4) favours a unit cell with a square base of side about 184 Å. As there are few unit cells (23 in a fibre of 1,000 Å diameter) in a fibre cross-section, the relative breadth of the equatorial intensities does not necessarily mean that crystallinity in the lateral plane is less than along the axis. Additionally, fibres of unlike diameters could have slightly different basal unit-cell dimensions which would also give rise to broadening.

Within each quadrant (Fig. 3), between the equator and meridian, there are two maxima. The stronger of these is situated at the intersection of the first layer and row lines, while the second, at a higher angle, has a meridional position between the second and third layer lines, thus the true axial repeat is $n \times 223$ Å where n is an integer. Its smallest possible value is 2, in which case the second off-meridional reflection, if well resolved (its position could be influenced by intensities on adjacent layer lines), should lie halfway between layer lines 2 and 3. Figure 3 shows that this is approximately so but the maximum is broad and weak and consequently its position cannot be determined unequivocally. The value of $n = 2$ is supported by micrographs⁷ and agrees with the prevalent view that the approximately 450 Å long molecules form a half-staggered arrangement along the fibre axis^{8,10}. Therefore this analysis, which is partly qualified as described above, shows that the diffraction pattern can be indexed on a tetragonal unit cell

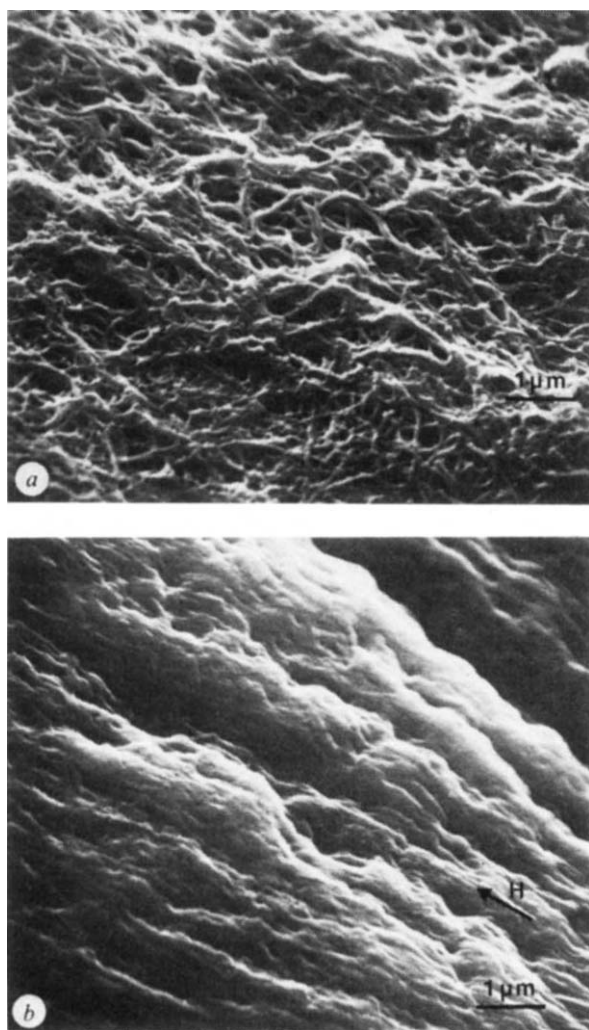


Fig. 2 Scanning electron micrographs of fibrin prepared as described in Fig. 1 legend. *a*, Without field; *b*, with field H in direction of arrow. The diameter of the fibres in *a* is $\sim 1,000$ Å. Drying has induced distortion and coalescence of the oriented *b* sample.

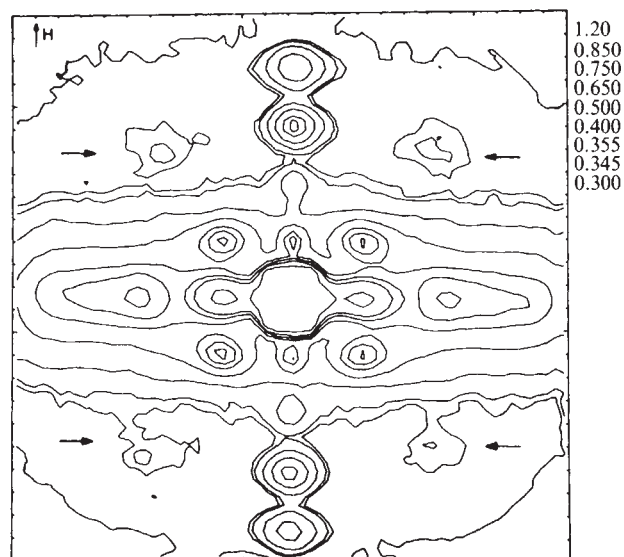


Fig. 3 Neutron diffraction pattern from fibrin (14 mg ml^{-1}) oriented in a magnetic field H of up to 18 T (arrow indicates field direction). The contour lines connect points of equal diffraction intensity. The conditions of the reaction are analogous to those described in Fig. 1 legend. The orientation was carried out in H_2O buffer which was replaced with D_2O buffer by diffusion, thereby greatly reducing the background level. The meridional reflections have a 223 ± 5 Å periodicity and the sharp equatorial peaks on either side of the centre are at 184 ± 5 Å. The arrows are drawn midway between the second and third layer lines. The central, rectangular region is due to the beam catcher. A sample in H_2O buffer gave a similar but less detailed diffraction pattern. Much slower polymerization in a field of 7 T also produced substantial orientation. The data were obtained with the two-dimensional (64×64 -cm) detector of the low-angle scattering instrument D11²⁶ at the Institut Laue-Langevin, Grenoble. The specimen-to-detector distance was 2.55 m, the wavelength 5.8 Å and the spread $\Delta\lambda/\lambda$ 8% (full width, half maximum). So far, X-ray patterns from gels formed as above have only shown the meridional reflections.

(with $a = 2 \times 185$ Å and $c = 446$ Å) that contains eight molecules (using 6 Å^3 per dalton for the specific volume¹³) and has a space group which restricts reflections to l even when $h = k = 0$. There are no evident restrictions on the general (h, k, l) reflections. None of the four space groups proposed in a recent model building study¹³ is supported by the diffraction pattern. However, one of these (space group I4) does have a tetragonal lattice with the unit-cell dimensions we find. Only the space groups $P4_2$ and $P4_22$ satisfy the above conditions.

The magnetic orientation demonstrated above is due to the diamagnetic anisotropy of the particles⁹. This depends strongly on the orientational order of the constituent diamagnetically anisotropic groups, which are chiefly α -helical peptide bonds and aromatic amino acid residues. Fibrin fibres orient parallel to the magnetic field and they contain a substantial proportion of α -helix¹⁴ ($\sim 30\%$ (refs 15, 16) by analogy with fibrinogen), which is also known to orient in the field direction^{9,17}. A preliminary conclusion, which will be justified more completely elsewhere, is therefore that the α -helical regions in fibrin are aligned relatively parallel to the fibre long axis.

Clearly magnetic field orientation is a valuable technique. This is demonstrated by the success in orienting cell membranes^{18,20} and two cylindrical viruses^{21,22}. The experiments reported here confirm that magnetic orientation of biological samples is increasingly viable²¹. The technique could be extended to other biopolymers and to living cells. Oriented polymers have previously been produced by the application of magnetic fields²³⁻²⁵, but in these the monomers formed a liquid-crystalline phase which oriented before polymerization advanced. In the formation of fibrin, the fibrinogen molecules (the precursors of monomers) interact very little, do not show

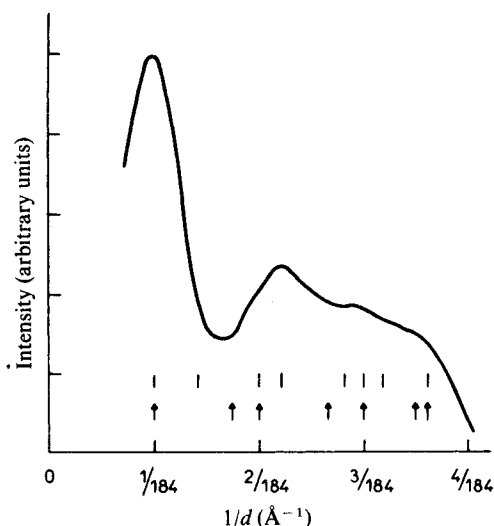


Fig. 4 The distribution of neutron-scattered intensity along the equator of Fig. 3; $1/d$ is the reciprocal of the Bragg spacing. The possible equatorial reflections from unit cells with a square (side 184 Å) and hexagonal (side $184 \times 2/3^{1/2}$ Å) base are shown by vertical lines and arrows respectively.

liquid-crystalline properties and orient poorly even in the strongest fields. These are important differences—they demonstrate that a liquid-crystalline phase or preformed orientation is not a prerequisite for the production of oriented polymers.

We thank G. Maret and C. Wilkinson for help and C. Berthet-Colominas for help with X-ray diffraction experiments. J.T. thanks the Deutsche Forschungsgemeinschaft for support. The work was partly supported by INSERM, FRA21. We are indebted to the Service National des Champs Intenses and to the Institut Laue-Langevin, both in Grenoble.

Received 28 July; accepted 24 October 1980.

1. Doolittle, R. F. *Adv. Protein Chem.* **27**, 1–109 (1973).
2. Doolittle, R. F. in *The Plasma Proteins* Vol. 2 (ed. Putnam, F. W.) 109–161 (Academic, New York, 1975).
3. Hall, C. E. & Slayter, H. S. *J. biophys. biochem. Cytol.* **5**, 11–15 (1959).
4. Fowler, W. E. & Erickson, H. P. *J. molec. Biol.* **134**, 241–249 (1979).
5. Tooney, N. M. & Cohen, C. *J. molec. Biol.* **110**, 363–385 (1977).
6. Weisel, J. W., Warren, G. S. & Cohen, C. *J. molec. Biol.* **126**, 159–183 (1978).
7. Krakow, W., Endres, G. F., Siegel, B. M. & Scheraga, H. A. *J. molec. Biol.* **71**, 95–103 (1972).
8. Ferry, J. D. *Proc. natn. Acad. Sci. U.S.A.* **38**, 566–569 (1952).
9. Worcester, D. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5475–5477 (1978).
10. Stryer, L., Cohen, C. & Langridge, R. *Nature* **197**, 793–794 (1963).
11. Conio, G., Dondero, G., Troglia, C., Trefiletti, V. & Patrone, E. *Biopolymers* **14**, 2363–2372 (1975).
12. Karges, H. E. & Kühn, K. *Eur. J. Biochem.* **14**, 94–97 (1970).
13. Hermans, J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1189–1193 (1979).
14. Bailey, K., Astbury, W. T. & Rudall, K. M. *Nature* **151**, 716–717 (1943).
15. Mihalyi, E. *Biochim. biophys. Acta* **102**, 487–499 (1965).
16. Budzynski, A. Z. *Biochim. biophys. Acta* **229**, 663–671 (1971).
17. Torbet, J. & Maret, G. *Biopolymers* (submitted).
18. Geacintov, N. E., Van Nostrand, R., Pope, M. & Tinkel, J. B. *Biochim. biophys. Acta* **226**, 486–491 (1972).
19. Saibil, H. R., Chabre, M. & Worcester, D. J. *Nature* **262**, 266–270 (1976).
20. Neugebauer, D.-Ch., Blaurock, A. E. & Worcester, D. L. *FEBS Lett.* **78**, 31–35 (1977).
21. Torbet, J. & Maret, G. *J. molec. Biol.* **134**, 843–845 (1979).
22. Maret, G. *et al.* in *Nonlinear Behaviour of Molecules, Atoms and Ions in Electric, Magnetic or Electromagnetic Fields* (Elsevier, Amsterdam, 1979).
23. Cser, F. *J. Phys. théor. appl.* **40**, 459–470 (1979).
24. Perplies, E., Ringsdorf, H. & Wendorf, J. H. *Polym. Lett. Ed.* **13**, 243–246 (1975).
25. Clough, S. B., Blumstein, A. & Hsu, E. C. *Macromolecules* **9**, 123–127 (1976).
26. Ibel, K. *J. appl. Crystallogr.* **9**, 630–643 (1976).

Orientation of oxygen in oxyhaemoproteins and its implications for haem catabolism

Stanley B. Brown*, Anthony A. Chabot†, Elizabeth A. Enderby* & Anthony C. T. North†

* Department of Biochemistry and † Astbury Department of Biophysics, University of Leeds, Leeds LS2 9JT, UK

Haem is degraded to bile pigments in the catabolism of haemoproteins in mammals¹ and in the formation of photosynthetic pigments in algae². The first stage of this reaction involves oxygen attack at one of the four methene-bridge carbon atoms, which is ultimately eliminated as CO (ref. 1). The four bridges are not sterically equivalent (Fig. 1) and the bilirubin in mammalian bile and algal bile pigments consists almost exclusively of the α -isomers. Little is known about the structures of the ring-cleaving enzymes responsible, although microsomal haem oxygenase, which catalyses the breakdown of haem to biliverdin in mammals³, has very similar spectroscopic properties to myoglobin³. The degradation process has been simulated *in vitro* by a 'coupled oxidation' method in which the proportions of the four possible isomeric products depend on the nature of the globin moiety to which the haem is bound⁴. We report here the use of an interactive computer display system⁵ to explore the relative accessibilities of the four methene bridges to a haem-bound oxygen molecule in myoglobin and in the α and β chains of haemoglobin. Our calculated interaction energies agree well with the proportions of the four isomers that are observed experimentally.

The coupled oxidation method, in which the haem compound is reacted with a reducing agent (for example, ascorbate or hydrazine) and molecular oxygen⁴, seems to be a good model for biological haem cleavage as the products (biliverdin, carbon

monoxide and free iron) are the same and a reducing agent (NADPH) and molecular oxygen are required *in vivo* also. Labelling experiments using ¹⁸O have confirmed the similarity of the mechanisms of oxygen incorporation into bile pigment in living rats⁶, algae⁷, in an *in vitro* haem oxygenase system⁸ and in various coupled oxidation systems^{8,9}.

Whereas coupled oxidation of free haem in aqueous pyridine produces an almost random mixture of the four isomers⁴, coupled oxidation of haemoproteins results in selective reactions; myoglobin, like haem oxygenase, yields α isomer almost exclusively, whereas human haemoglobin yields 65% α isomer and 35% β isomer with little or no γ or δ isomer⁴. After examination of molecular models of haemoglobin¹⁰ and myoglobin¹¹, Brown¹² suggested an intramolecular mechanism for the activation of molecular oxygen for haem degradation and for the determination of bridge selectivity, an essential feature of which is attack of a methene bridge carbon atom by an iron-bound oxygen molecule, considered to be liganded as in oxyhaemoglobin or oxymyoglobin. In protein-free haem, each methene bridge would be equally accessible—this would account for the random formation of biliverdin isomers. In haemoproteins, access might be restricted by the limited space in the haem pocket, and the molecular models suggested that variations in accessibility were broadly consistent with the ratios of products observed.

We attempted to evaluate this intramolecular degradation hypothesis in a more quantitative way by calculating the energies of interaction between a haem-bound oxygen molecule and the surrounding protein residues as the oxygen molecule is rotated about the Fe-O axis (Fig. 1). We used an interactive computer display system⁵ in which the present molecular conformation was depicted on a cathode ray tube as it was adjusted, and which could also evaluate functions that depend on conformation, such as the potential energy due to dispersion forces between pairs of atoms: this we modelled by the expression $E = -a_{ij}r_{ij}^{-6} + b_{ij}r_{ij}^{-12}$, where r_{ij} is the distance between atoms i and j and a_{ij} , b_{ij} are constants characteristic of the types of atoms concerned¹³. We evaluated E at 5° steps of the torsion angle τ

which defines rotation of $O_A O_B$ about the FeO_A bond, including contributions for all significant pairwise interactions that involve O_B , apart from those with Fe and with O_A , which are independent of τ . Energies for the two kinds of haemoglobin chain were evaluated in isolation because interactions between O_B and atoms in neighbouring subunits are insignificant. Hydrogen atoms were not included in our calculations as experience suggests that they have relatively little effect due to the 'softness' of their potential function and the ability of methyl groups to rotate so as to minimize steric hindrance.

For haemoglobin, the X-ray coordinates of the oxy derivative are not known; therefore we used those published for horse aquo-methaemoglobin, apart from those of the Fe atom. Horse and human haemoglobins have identical amino acid sequences in the haem pocket region except for the substitution of β -E14 Ser (horse) by Ala (human); we allowed for this substitution by omitting the hydroxyl group, although this residue is too remote for it to contribute significantly to the interaction with O_B . Similarly, for myoglobin we used the published coordinates of sperm whale metmyoglobin apart from those of the Fe atom. These globin coordinates were obtained from the Protein Data Bank¹⁴, files 2MHB and 1MBN respectively. The coordinates of each of these structures had been refined with constraints that forced the atoms of the haem groups (apart from the pendant side chains) to be coplanar and 4-fold symmetrical. We placed the Fe atoms on the axis of 4-fold symmetry but displaced from the plane of the haem group towards the proximal histidine residue by the distances given by Phillips¹⁵: 0.033 nm for myoglobin, 0.007 nm for the haemoglobin α chain and 0.021 nm for the β chain. For each chain, we used Phillips' values of 0.19 nm for FeO_A , 0.14 nm for $O_A O_B$ and 121° for the angle $FeO_A O_B$.

The energy values thus calculated (Fig. 2 a-c) indicate the relative probability of finding the oxygen molecule in any particular orientation τ about the haem normal; the higher the energy, the less likely is the oxygen to occupy that position. The most probable position would correspond to the energy minimum, but because we are interested in the likelihood of the oxygen being aligned so as to attack one of the four methene-bridge carbon atoms, our primary concern is with the relative energies in the neighbourhoods of the four corresponding values of τ .

For myoglobin (Fig. 2a) the energy shows a pronounced maximum at about 325° and at the γ bridge the energy is $105 \text{ kcal mol}^{-1}$ above the minimum. This high energy is due to the presence of the distal His residue (E7) which renders the γ bridge completely inaccessible to the bound oxygen molecule. Of the other three bridge positions, α has by far the lowest

Table 1 Contributions made by subunits of haemoproteins to isomers formed by coupled oxidation

Haemoprotein	% Isomer formed by coupled oxidation				Energy above lowest minimum (kcal mol^{-1})			
	α	β	γ	δ	α	β	γ	δ
Sperm whale myoglobin	100	—	—	—	0.25	14.1	105	9.6
Human haemoglobin (α subunits)	55	45	—	—	0	0.6	129	10.8
Human haemoglobin (β subunits)	75	25	—	—	0	2.0	1,240	7.7

Results for α and β subunits are from ref. 160

energy (Table 1) as it is only 10° away from the minimum in a relatively flat part of the curve. The energies corresponding to the δ and β positions are much higher because of interactions with E11 Val and CD1 Phe respectively, suggesting that the β and δ orientations would be adopted only by a very few molecules. Thus, degradation of the α methene bridge would be very strongly preferred over degradation of the other bridges, which agrees with experiments.

The profile for the haemoglobin α chain (Fig. 2b) is grossly similar to that for myoglobin with a very high barrier due to the distal His and a relatively high energy corresponding to the δ bridge. The low energy region from $\tau \sim 160$ to $\tau = 280^\circ$ is much broader than in myoglobin and encompasses both the α and β positions. The actual minimum is exactly at the α bridge but the energy at the β position is only $0.6 \text{ kcal mol}^{-1}$ higher. This suggests that both α and β bridges would be susceptible to attack, with the α bridge marginally favoured.

For the β subunits also, the γ and δ positions show a very high energy and the lowest minimum occurs precisely at the α bridge. The region between $\tau = 160$ and $\tau = 280^\circ$ is different in profile from that for the α subunit, with an energy barrier between the α and β methene bridges. At the β bridge, the energy is 2 kcal mol^{-1} above that at the α bridge, so that more α isomer and less β isomer would be formed from haemoglobin β chains than from α chains. However, taken together, our calculations are clearly consistent with the experimental observation that the biliverdin produced by coupled oxidation of whole haemoglobin contains 65% α and 35% β isomer.

While these calculations were being carried out, Docherty¹⁶ determined the separate contributions made by the α and β subunits to the isomer pattern obtained by coupled oxidation of haemoglobin. His results (Table 1), which showed that the β subunits produce some β isomer but not as much as do the α subunits, conformed well with our predictions.

Our observations strongly favour the intramolecular theory of haem degradation both in explaining the absence of γ and δ isomers and in accounting for the variation in proportion of the α and β isomers. Further support has been obtained from studies of the coupled oxidation of some abnormal haemoglobins¹⁷. For example, in haemoglobin Zürich, E7 His (the residue that blocks access to the γ bridge) is replaced by Arg, which is displaced from the haem pocket. We therefore predict that the γ methene bridge might be accessible to attack, which agrees with the experimental result that the γ isomer is present in the products.

Our energy profiles vary rapidly in the region of the β bridge so that relatively small changes in protein structure can greatly affect the amount of β isomer produced; this may explain the variations observed in the amount of β isomer in the degradation products of animal haemoglobins (S.B.B., unpublished) and plant leghaemoglobins¹⁸.

There are several uncertainties in our calculations which cannot be readily resolved: (1) the coordinates used refer strictly to the aquo-met forms of the haemoproteins rather than the oxy forms; (2) the geometry of the oxygen molecule in the transition state of the reaction may differ from that assumed; (3) the

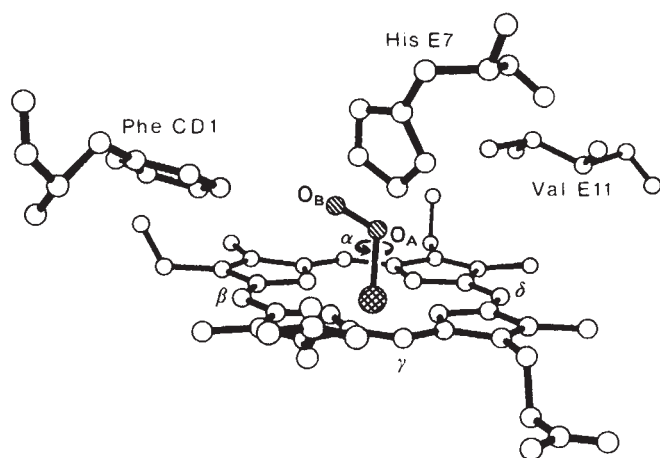


Fig. 1 Oxygen binding site in globins. The side chains shown are those primarily involved in interactions with the oxygen molecule and occur in generally similar, though not identical, positions in the three globin chains studied. The arrow indicates the torsion angle τ through which the oxygen molecule is rotated about the $Fe-O_A$ bond. The four methene bridges are labelled α , β , γ , δ .

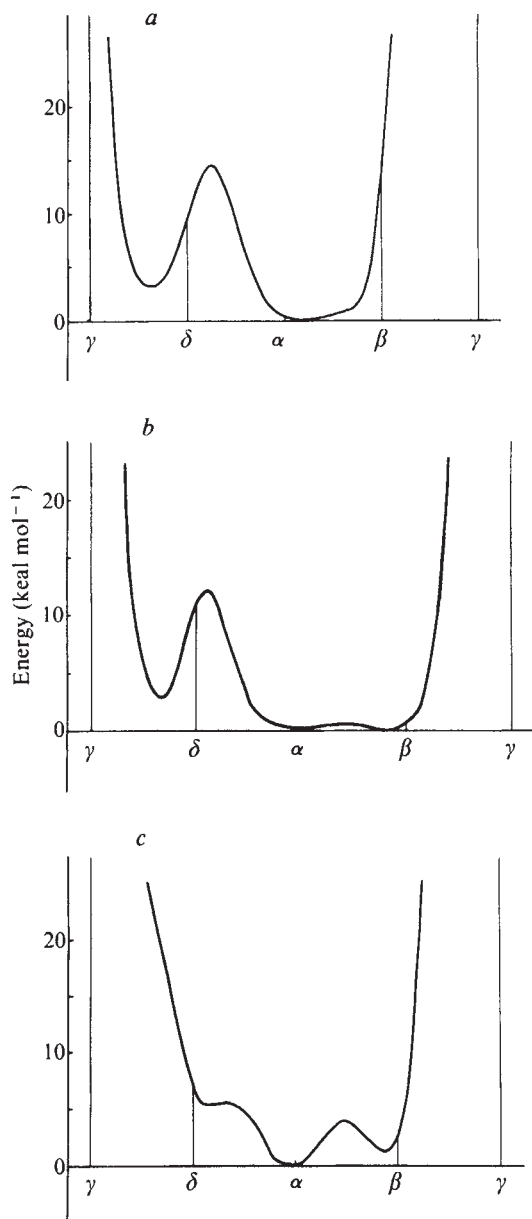


Fig. 2 Variation of interaction energy with rotational angle τ for several haemoproteins. The energy values refer to an arbitrary zero corresponding to the minimum in each case. Rotation is such that the α , β , γ and δ bridges correspond to τ values of 180, 270, 360(0°) and 90° respectively. *a*, Sperm whale myoglobin; *b*, human haemoglobin (α subunits); *c*, human haemoglobin (β subunits).

oxygen may be suitably disposed to attack a methene bridge within a range of τ values, necessitating integration of the energy functions; (4) we ignored electrostatic interactions and (5) it is now known (S.E.V. Phillips, personal communication) that the haem rings are not coplanar but rather puckered. Clearly, we would not have obtained the present level of correlation if these effects were too large, but we would be cautious about a more quantitative interpretation until data on other haemoproteins become available.

Finally, in all three haemoprotein chains studied, the minimum energy position is coincident with or close to the position of the α methene bridges. As there is no intrinsic reason that the energy minimum should correspond to any of the methene bridges, we wonder whether the situation is advantageous. It seems unlikely, although not impossible, that this feature of haemoproteins has evolved to facilitate the production of α isomers of bile pigments after degradation. Perhaps an 'open' α

methene-bridge region may favour access of oxygen to the iron atom in the normal reversible interaction of haemoproteins with oxygen.

We thank the MRC for the award of a Project Grant to S.B.B. and the SRC for support to A.C.T.N. A.A.C. was supported by a Wellcome Research Studentship.

Received 25 July; accepted 10 November 1980.

- Schmid, R. & McDonagh, A. F. *Ann. N.Y. Acad. Sci.* **244**, 533–552 (1975).
- Troxler, R. F. & Bogorad, L. *Pl. Physiol.* **41**, 491–499 (1966).
- Yoshida, T. & Kikuchi, G. *J. biol. Chem.* **254**, 4487–4491 (1979).
- O'Carra, P. in *Porphyrins and Metalloporphyrins* (ed. Smith, K. M.) 123–153 (Elsevier, Amsterdam, 1975).
- North, A. C. T., Denson, A. K., Evans, A. C., Ford, L. O. & Willoughby, T. V. in *Biomolecular Structure, Conformation, Function and Evolution* Vol. 1 (ed. Srinivasan, R.) 59–72 (Pergamon, Oxford, 1980).
- Brown, S. B. & King, R. F. G. *J. Biochem. J.* **170**, 299–311 (1978).
- Troxler, R. F., Brown, A. S. & Brown, S. B. *J. biol. Chem.* **254**, 3411–3418 (1979).
- King, R. F. G. & Brown, S. B. *Biochem. J.* **174**, 103–109 (1978).
- Chaney, B. D. & Brown, S. B. *Biochem. Soc. Trans.* **6**, 419–421 (1978).
- Ladner, R. C., Heidner, E. G. & Perutz, M. F. *J. molec. Biol.* **114**, 385–414 (1977).
- Kendrew, J. C. & Watson, H. C. *Prog. Stereochem.* **4**, 299–333 (1969).
- Brown, S. B. *Biochem. J.* **159**, 23–27 (1976).
- Scott, R. A. & Scheraga, H. A. *J. chem. Phys.* **45**, 2091–2101 (1966).
- Bernstein, F. C. *et al. J. molec. Biol.* **112**, 535–542 (1977).
- Phillips, S. E. V. *Nature* **273**, 247–248 (1978).
- Docherty, J. C. thesis, Univ. Leeds (1980).
- Brown, S. B. & Docherty, J. C. *Biochem. J.* **173**, 985–987 (1978).
- Lehtovaara, P. & Perttala, U. *Biochem. J.* **176**, 359–364 (1978).

Are archaebacteria merely derived 'prokaryotes'?

Carl R. Woese & Ramesh Gupta

Department of Genetics and Development, 515 Morrill Hall, University of Illinois, Urbana, Illinois 61801

The archaebacteria are a group of prokaryotes which seem as distinct from the true bacteria (eubacteria) as they are from eukaryotes^{1–4}. The evidence on which this conclusion rests is of two types: genotypic (quantitative)—that is, comparative sequence studies, and phenotypic (qualitative)—that is, differences in various organismal characteristics. The differences between archaebacteria and true bacteria are so great, both quantitatively and qualitatively, that the two bacterial groups should be considered as representing separate primary lines of descent, each tracing directly back to the universal ancestor^{1,4}. Furthermore, this ancestor itself seems not to be a prokaryote; rather it was a far simpler type of organism, one properly called a progenote^{5,6}. If this is true, the discovery of archaebacteria marks a major advance in the biologist's attempts to understand the basis for the evolution of the cell.

Recently van Valen and Maiorana have challenged this interpretation⁷—they consider that the archaebacteria represent merely one branch of a general prokaryotic phylogenetic tree. Their interpretation requires the archaebacteria to have undergone more extensive evolution, both quantitatively and qualitatively, than have other prokaryotes. They also claim that archaebacteria are the forerunners of the so-called eukaryotic 'host cell', the presumed entity which housed the endosymbionts that later became eukaryotic mitochondria, chloroplasts, etc.⁷. Their interpretation is a conservative one—it seeks to retain the conventional prejudices regarding the evolution of bacteria and the origins of the eukaryotic cell (for example, prokaryotes are monophyletic; fermentative metabolism preceded autotrophic or phototrophic metabolism in prokaryotes; there are only two basic types of living system on Earth, eukaryotes and prokaryotes; the eukaryotic ancestor was a prokaryote that lost its cell wall^{1,7,8–10}). A clearcut choice between the conservative and the radical interpretation of the facts cannot be made without more evidence than now exists. Our purpose in discussing the two interpretations, therefore, is

not to eliminate one, but to stress the differences between them, thus preparing the ground for a future decision when more becomes known about the archaeobacteria.

First, consider the genealogical relationship of archaeobacteria to true bacteria ('prokaryotes' as the molecular biologist defines them). The issue is whether archaeobacteria constitute a line of descent within the phylogenetic tree defined by the true bacteria⁷, or whether the archaeobacterial line branched from the common stem at a point before the ancestor of all true bacteria had evolved, and if the latter, whether the ancestor common to true bacteria and archaeobacteria was itself a prokaryote or a simpler form, a progenote in our terminology^{1,6}. Are prokaryotes of monophyletic or polyphyletic origin?

Those properties shared by (most) true bacteria must be largely ancestral. When defined in moderate detail many of these presumed ancestral properties are found to be absent in archaeobacteria, which instead have different but equivalent properties. The conservative view is that archaeobacteria have lost these ancestral 'prokaryotic' properties and have re-evolved their equivalents: for example, archaeobacteria (on the basis of this conservative view) would have had to lose the typical bacterial cell wall and evolved new types of walls^{3,5,11,12}; have drastically altered the characteristic sequence of 'prokaryotic' rRNA (ref. 3); changed the characteristic pattern for nucleotide modification in both rRNA and tRNA^{2,4,13}; evolved ether-linked, branched chain lipids, discarding ester-linked, straight chain lipids in the process^{14,15}; changed the subunit structure of RNA polymerase^{16,17}; and they would have had to discard many of the antibiotic sensitivities that are typical of prokaryotes and of eukaryotic organelles, again acquiring different ones². Were archaeobacteria as well characterized as are the true bacteria, this list of changes in true bacterial characteristics, ostensibly wrought during archaeobacterial evolution, might be enormous. If we extrapolate from the little knowledge we have of archaeobacteria, it seems that true bacteria and archaeobacteria differ significantly in the details of almost all molecular processes^{2,4}.

The conservative explanation attempts to account for the many molecular disparities between archaeobacteria and true bacteria by invoking a period of 'rapid evolution' at an early stage in the line of archaeobacterial descent⁷. This explanation is too plastic and ill-defined; seemingly any outcome can be rationalized. Peculiarities of archaeobacterial niches alone could not seemingly account for this supposed rapid evolution. Species of eubacteria cohabit some niches with archaeobacteria; for the former, there is evidence of only minor, local changes in molecular processes as a result of adaptations¹⁸. Examples of apparently rapid evolution among true bacteria have been encountered—the true mycoplasmas are one. (These organisms are actually highly derived offshoots of the clostridia¹⁹.) Although they are unique, the mycoplasmas retain a great deal more genotypic resemblance to true bacteria than do the archaeobacteria¹⁹. The eukaryotic organelles are another example^{20,21}—in the case of the animal mitochondrion perhaps more drastic than might be expected from a free-living organism. However, even the highly idiosyncratic animal mitochondrion, which has the least rRNA sequence homology with eubacterial rRNAs of all organisms^{3,20}, shows a marked resemblance to true bacteria—for example, in the details of its respiratory chain²², antibiotic sensitivities²³, modified nucleotides in tRNA²⁴, and perhaps in the secondary structure of its rRNA (H. F. Noller and C. R. Woese, unpublished).

The issue here is the extent of the differences between archaeobacteria and true bacteria. Is it possible that once the cell's basic molecular processes have been established (and are well integrated into the fabric of the cell), that an organism can proceed along an evolutionary course and encounter niches that cause it to alter almost all these processes in significant ways? We consider the amount of change that can be effected in the basic molecular processes once they are established is quite limited, and if our present knowledge of archaeobacteria does not exceed these limits, then our future knowledge of

them certainly will. For this reason, it has been suggested that the archaeobacterial and true bacterial lines of descent separated from one another during the stage at which the cell's basic molecular processes were still evolving and being refined^{4,6}. Each line separately completed the evolution of the various molecular functions, as it evolved from the progenote to the prokaryotic state¹. (One of the predictions of the radical view is that archaeobacteria should possess very different genetic control mechanisms from true bacteria^{2,6}, in that genetic control is largely a series of refinements in basic gene expression mechanisms.)

The conservative interpretation is that the archaeobacterial line of descent gave rise to the eukaryotic cell, that is, the putative host in which the endosymbionts reside⁷. It is not unreasonable that archaeobacterial genes and structures are found in the chimaera that is the eukaryotic cell, like true bacterial genes and structures are⁴ (C.R.W., in preparation). In fact, one of the archaeobacterial ribosomal proteins shows a distinct specific sequence homology with its eukaryotic counterpart²⁵. Archaeobacteria do share a number of properties specifically with eukaryotes^{2,4,26,27}. However, we consider that such a suggestion is unwarranted, for several reasons. Few facts are known and they do not strongly support the argument. Archaeobacteria have as many properties in common with true bacteria as they do with eukaryotes, and true bacteria also share properties specifically with eukaryotes^{2,28,29}; also the eukaryotic 18S rRNA seems to be no more closely related to its archaeobacterial than its eubacterial counterpart^{1,4}. Furthermore, archaeobacteria already have a disturbing number of dissimilarities to eukaryotes². Too little is known about the way in which eukaryotic cells evolved to justify this specific conclusion⁷—we do not know enough about genealogies of various eukaryotic (and bacterial) genes. We are not even certain that there was such a thing as the ancestral eukaryotic 'host cell', as it is now envisaged^{7,8}. We do not know the extent to which endosymbioses are responsible for the chimaeric condition of the eukaryotic nucleus (C.R.W., in preparation). It is more useful at this stage to state merely that archaeobacteria as well as true bacteria seem to be represented in the eukaryotic chimaera, and that other lines of descent, other groups, may be represented there as well (for example, the 18S rRNA)^{2,4}. The ways in which the various lines are so represented, the evolutionary stages at which the associations occurred and the number of such events involved are all open questions, to be decided by comparative molecular analyses of eukaryotes, eubacteria and archaeobacteria.

Received 9 October; accepted 10 November 1980.

- Woese, C. R. & Fox, G. E. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5088–5090 (1977).
- Woese, C. R., Magrum, L. J. & Fox, G. E. *J. molec. Evol.* **11**, 245–252 (1978).
- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. *Microbiol. Rev.* **43**, 260–296 (1979).
- Fox, G. E. *et al. Science* **209**, 457–463 (1980).
- Woese, C. R. *Symp. Soc. gen. Microbiol.* **20**, 39–54 (1970).
- Woese, C. R. & Fox, G. E. *J. molec. Evol.* **10**, 1–6 (1977).
- VanValen, L. M. & Maiorana, V. C. *Nature* **287**, 248–250 (1980).
- Margulis, L. *Origin of Eucaryotic Cells* (Yale University Press, New Haven, 1970).
- Stanier, R. Y. *Symp. Soc. gen. Microbiol.* **20**, 1–38 (1970).
- Murray, R. G. E. in *Bergey's Manual of Determinative Bacteriology* 8th edn (eds Buchanan, R. E. & Gibbons, N. E.) (Williams & Wilkins, Baltimore, 1974).
- Kandler, O. & König, H. *Archs Microbiol.* **118**, 141–152 (1978).
- Kandler, O. *Naturwissenschaften* **66**, 95–107 (1979).
- Gupta, R. & Woese, C. R. *Curr. Microbiol.* (in the press).
- Langworthy, T. A. *Biochim. biophys. Acta* **487**, 37–47 (1977).
- Tornabene, T. G. & Langworthy, T. A. *Science* **203**, 51–53 (1979).
- Zillig, W., Stetter, K. O. & Janekovic, P. *Eur. J. Biochem.* **96**, 597–604 (1979).
- Zillig, W., Stetter, K. O. & Tobein, M. *Eur. J. Biochem.* **91**, 193–199 (1978).
- Langworthy, T. A., Mayberry, W. R. & Smith, P. F. *Biochim. biophys. Acta* **431**, 550–569 (1976).
- Woese, C. R., Maniloff, J. & Zablen, L. B. *Proc. natn. Acad. Sci. U.S.A.* **77**, 494–498 (1980).
- Eperon, I. C., Anderson, S. & Nierlich, D. P. *Nature* **286**, 460–467 (1980).
- Bonen, L. & Doolittle, W. F. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2310–2314 (1975).
- John, P. & Whately, F. R. *Nature* **254**, 495–498 (1975).
- Flavell, R. *Biochem. Genet.* **6**, 275–291 (1972).
- Heckman, J. E. *et al. Cell* **13**, 83–95 (1978).
- Matheson, A. T., Moller, W., Amos, R. & Yaguchi, M. in *Ribosomes: Structure, Function, and Genetics* (eds Chamblis, G. *et al.*) 297–315 (University Park Press, Baltimore, 1980).
- Kessel, M. & Klink, F. *Nature* **287**, 250–251 (1980).
- Kwok, Y. & Wong, J. T-F. *Can. J. Biochem.* **58**, 213–218 (1980).
- Steitz, J. A. *Nature* **273**, 10 (1978).
- Woese, C. R. & Fox, G. E. *Nature* **273**, 101 (1978).

MATTERS ARISING

Pyroclastic sulphur

FRANCIS *ET AL.*¹ recently commented on the apparent rarity of pyroclastic sulphur in terrestrial eruptions. They described sulphur-rich ejecta from Poás volcano, Costa Rica, and mentioned the presence of myriads of small, sulphur spheres. Similar spheres have been reported in tephra erupted from Ruapehu²⁻⁴, New Zealand, and may well be common at many volcanoes where an active vent is occupied by a crater lake.

The Ruapehu spheres range from 50 μm to 1 mm, but most are 125–750 μm in diameter. They have a lustrous, granular surface, and comprise solid, black, orthorhombic sulphur with disseminated silicate, and iron sulphide dust, a few small vacuoles and rare, yellow sulphur crystals. Some are globular, some are fused double-spheres, but most are almost perfectly round 'rough' spheres'. They were ejected in abundance by phreatomagmatic eruptions in 1971, and one fine ash collected 15 km downwind comprised 12% by volume of spheres². Sulphur also occurs in Ruapehu tephra as uncommon, angular, yellow fragments, and sulphur drip on ejected blocks. Rafts of spongy sulphur float on the lake in quiet or eruptive periods.

Vent morphology and activity of Ruapehu⁵ and Poás appear similar, and a subaqueous liquid sulphur lake has been postulated for both volcanoes^{7,8}. I agree with Francis *et al.*¹ that this is unlikely. Much consolidated, volcanoclastic/chemogenic sediment has been erupted from Ruapehu, and in some siltstones ejected in 1971, sulphur occurs as globules in cavities, and veins crossing the bedding. This I believe is evidence that sulphur is 'sweated' out of sulphurous sediment, whence it may enter superheated steam fumaroles and be discharged violently into muddy lake water to form black droplets. The lack of coarse, clastic or scoriaceous sulphur suggests that there were no large accumulations of liquid sulphur present when the lake bed was fragmented during phreatomagmatic eruptions of Ruapehu.

C. P. WOOD

New Zealand Geological Survey,
PO Box 30368,
Lower Hutt,
New Zealand

7. Giggenbach, W. *N.Z. J. Sci.* **17**, 33–45 (1974).
8. Bennett, F. D. & Raccichini, S. M. *Nature* **271**, 342–344 (1978).

FRANCIS REPLIES—We were not aware of the existence of small sulphur spherules in the material ejected by the Ruapehu eruption of 1971, and are grateful to Wood for bringing it to our attention. We agree that such spheres may well be common in active volcanoes with crater lakes, and would welcome any further observations of these and related phenomena.

P. W. FRANCIS

Department of Earth Sciences,
The Open University,
Walton Hall,
Milton Keynes MK7 6AA, UK

Recombination intermediates

IN the summary of their elegant demonstration of adenovirus recombination intermediates, Wolgemuth and Hsu¹ state: "To the best of our knowledge, this is the first visualization of *in vivo* recombination intermediates of discrete DNA molecules isolated from eukaryotic cells". They appear to have overlooked the paper published last year by Bell and Byers² which presented an equally clear demonstration of Holliday-type recombination intermediates involving the 2- μm plasmids of *Saccharomyces cerevisiae*. Although this earlier case might be held to be atypical of recombination in general, in that it involved inverted repeats within the same molecule rather than homologous regions of different molecules, it was, in another respect, more relevant to meiotic recombination, because the Holliday structures were found specifically in cells at the prophase stage of meiosis.

J. R. S. FINCHAM

Department of Genetics,
University of Edinburgh,
Edinburgh EH9 3JN, UK

1. Wolgemuth, D. J. & Hsu, M.-T. *Nature* **287**, 168–171 (1980).
2. Bell, L. & Byers, B. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3445–3449 (1979).

WOLGEMUTH AND HSU REPLY—As stated in the text of our paper, our study does indeed represent the first example, to the best of our knowledge, of visualization of *in vivo* recombination intermediates in animal cells and we are sorry that the word eukaryotic in the summary was misleading. We would like to emphasize

Fincham's point that our observations are more typical of recombination in that they are inter- rather than intramolecular. Our failure to cite the paper by Beck and Byers in our discussion was totally inadvertent and we had contacted them previously in this regard.

DEBRA J. WOLGEMUTH
MING-TA HSU

The Rockefeller University,
1230 York Avenue,
New York, New York 10021

Common ancestry for birds and crocodiles?

A PSEUDOSUCHIAN origin for birds and crocodiles, independent of dinosaurs, has been proposed by Whetstone and Martin¹. They described two 'derived' characters of the otic region of the skull, a fenestra pseudorotunda and periotic sinuses, which were claimed to be unique to crocodiles and birds and thus evidence for their common ancestry. It is well known that crocodiles and birds possess a similar otic morphology^{2,3}. Any hypothesis of their common ancestry independent of dinosaurs therefore depends critically on the assertion that dinosaurs do not have similar shared features, or that if they do, the features are not homologous. In this respect, we think that Whetstone and Martin's evidence is equivocal and should be treated with caution.

Whetstone and Martin note that in primitive reptiles the perilymphatic duct exits from the cranium through the vagus foramen (fissura metotica), accompanied by certain cranial nerves. In crocodiles and birds, however, the perilymphatic duct has a separate exit, and terminates in a small membrane that closes the fenestra rotunda, or, more correctly, the fenestra pseudorotunda². The fenestra pseudorotunda lies very close to the fenestra ovalis.

Based on their interpretations of casts of the inner ear structure, Whetstone and Martin concluded that dinosaurs did not have a fenestra pseudorotunda. Casts of four of the seven genera studied were illustrated: the sauropod *Brachiosaurus*, the hadrosaur *Lophorhynchus*, the ankylosaur *Ankylosaurus* and the theropod *Allosaurus* (see ref. 1, Fig. 2 a–d, respectively). We question how the authors can be certain that the single trunk identified in *Lophorhynchus* and *Ankylosaurus* does in fact represent both the perilymphatic duct and certain cranial nerves. Why should it not represent the perilymphatic duct alone? In *Lophorhynchus*, for example, could not the first trunk (labelled perilymphatic duct plus nerves X–XI) represent the perilymphatic duct exiting alone, and

1. Francis, P. W., Thorpe, R. S., Brown, G. C. & Glasscock, J. *Nature* **283**, 754–756 (1980).
2. Wood, C. P. *N.Z. Volcan. Rec.* **1**, 22–24 (1973).
3. Wood, C. P. *N.Z. D.S.I.R. Bull.* **218**, 103–108 (1977).
4. Wood, C. P. *N.Z. D.S.I.R. Bull.* **224**, 35–39 (1978).
5. Walker, G. P. L., Wilson, L. & Howell, E. L. *J. Geophys. J. R. astr. Soc.* **22**, 377–383 (1971).
6. Cole, J. W. & Nairn, I. A. *Catalogue of the Active Volcanoes and Solfataral Fields of New Zealand* (IAVCEI, Rome, 1975).

ending in the fenestra pseudorotunda? The next trunk could then represent nerves X and XI and the third trunk nerve XII. The point here is that Whetstone and Martin's interpretation of a complex morphological structure from casts is questionable, and alternative explanations are possible.

Notwithstanding these questions of interpretation, which simply reflect the shortcomings of attempts to reconstruct the soft anatomy of fossil organisms, it is interesting that other workers have reported a fenestra pseudorotunda in the theropods *Dromaeosaurus*⁴ and *Gallimimus*⁵, and in the pachycephalosaurians *Prenocephale* and *Homalocephale*⁶. In *Dromaeosaurus*⁴ remnants of the stapes confirm the interpretation of the fenestra ovalis, and the adjacent aperture, identified as the fenestra (pseudo) rotunda (though identified as the IX foramen by Whetstone and Martin), bears a relationship with the fenestra ovalis which is strikingly similar to that illustrated by Whetstone and Martin for *Hesperornis* and *Alligator* (compare ref. 4, Fig. 7a, and ref. 1, Figs. 3a,b).

Whetstone and Martin marshalled evidence from embryology to support their case that the 'round window' (fenestra pseudorotunda) of birds and crocodiles is a homologous feature unique to them. Although other living vertebrates (mammals and lizards) also have round windows, they argued that the structure had a similar embryology in birds and crocodiles, and that this differed from that of other vertebrates. However, if some dinosaurs do indeed have a fenestra pseudorotunda, then the embryological evidence in support of its homology in birds and crocodiles is less compelling. The similar embryology of the otic regions of crocodiles and birds may only reflect their archosaurian ancestry.

The other character described by Whetstone and Martin, periotic sinuses associated with bones of the middle ear cavity, also may not be unique to crocodiles and birds. Depressions have been described in the lateral wall of the braincase of *Saurornithoides*⁷ that seem to be associated with the middle ear.

Thus, we find that the evidence presented by Whetstone and Martin to support an hypothesis of common ancestry of crocodiles and birds independent of dinosaurs is equivocal. It is sobering to recount Baird's⁸ summary statement in 1970: 'Parallelism and convergence in the tympanic regions of living reptiles are common, and only a few reptilian ears are yet known in detail.' Thus, even if Whetstone and Martin are correct in their assertion that a fenestra pseudorotunda and periotic sinuses are unique to birds and crocodiles, it remains to be determined whether this is due to common ancestry or convergence. In view of the extensive evidence compiled by Ostrom⁹⁻¹⁴ for common ancestry of birds

and dinosaurs, the otic features mentioned by Whetstone and Martin may best be ascribed to homoplasy.

C. MCGOWAN

Department of Vertebrate
Palaeontology,
Royal Ontario Museum,
Toronto, Ontario,
Canada MS5 2C6 and
Department of Zoology,
University of Toronto,
Toronto, Ontario,
Canada MS5 1A1

A. J. BAKER

Department of Ornithology,
Royal Ontario Museum,
Toronto, Ontario,
Canada MS5 2C6
and Department of Zoology,
University of Toronto,
Toronto, Ontario,
Canada MS5 1A1

1. Whetstone, K. N. & Martin, L. D. *Nature* **279**, 234-236 (1979).
2. de Beer, G. R. *The Development of the Vertebrate Skull* (Oxford University Press, 1937).
3. Romer, A. S. & Parsons, T. S. *The Vertebrate Body* (Saunders, Philadelphia, 1977).
4. Colbert, E. H. & Russell, D. A. *Am. Mus. Nov.* No. 2380, 1-49 (1969).
5. Osmólska, H., Roniewicz, E. & Barsbold, R. *Palaeont. pol.* **27**, 103-143 (1972).
6. Maryanska, T. & Osmólska, H. *Palaeont. pol.* **30**, 46-102 (1974).
7. Barsbold, R. *Palaeont. pol.* **30**, 5-22 (1974).
8. Baird, I. L. in *Biology of the Reptilia: Morphology B*. Vol. 2 (eds Gans, C. & Parsons, T. S.) 193-275 (Academic, London, 1970).
9. Ostrom, J. H. *Nature*, **242**, 136 (1973).
10. Ostrom, J. H. *Q. Rev. Biol.* **49**, 27-47 (1974).
11. Ostrom, J. H. *A. Rev. Earth planet. Sci.* **3**, 55-57 (1975).
12. Ostrom, J. H. *Proc. Centre natn. Rech. Sci.* **218**, 519-532 (1975).
13. Ostrom, J. H. *Smithsonian Contr. Paleobiol.* **27**, 1-21 (1976).
14. Ostrom, J. H. *Biol. J. Linn. Soc., Lond.* **8**, 91-182 (1976).

WHETSTONE AND MARTIN REPLY— The fundamental difference between the otic capsules of *Sphenodon* and those of a bird or crocodile is in the course of the perilymphatic duct. In *Sphenodon*, it turns medially, enters the endocranium and passes along a groove before being exposed to pharyngeal tissues¹. In birds and crocodiles, it turns laterally and is exposed to the tympanic cavity. Either condition requires a correlated suite of osteological structures. We have used these structures in attempting to determine the course of the duct in dinosaurs². Hadrosaurs and *Ankylosaurus*, for example, are like *Sphenodon* but unlike birds or crocodiles, in having an open 'vestibule' to the endocranium, a groove between the vestibule and vagus foramen, and no confluence between the external cranial openings. Whether or not the duct left the cranium alone (as suggested by McGowan and Baker) is irrelevant to the problem at hand. Either interpretation presumes a primitive (medial) course for the duct, along the braincase wall.

By using a bird or crocodile as a model for fossil archosaurs, some workers have

restores a fenestra pseudorotunda in dinosaurs. The foramen indentified³ as the 'round window' in *Gallimimus* is not on the otic capsule, but is far up on the parocciput. In the type of *Dromaeosaurus*, the right otic capsule has been destroyed, along with the footplate of the stapes. The resulting hole and the remnants of a nerve canal were labelled 'Foramen Rotundum' in the original restoration⁴. The ear regions of the cited pachycephalosaurs are indeterminate. The best candidate for the presence of this structure is the ornithischian, *Hyposilophodon*. Were a 'window' present in dinosaurs, it would not lessen the embryological evidence for the homology of the fenestra in birds and crocodiles, but might indicate that the homology evolved in a more remote ancestor.

Ostrom's evidence for a theropod origin for birds has been well summarized⁵. The evidence for a 'sister-group' relationship to crocodilians, first suggested by Walker⁶, is still being developed by Whetstone, but the following derived features seem to be homologous: a fenestra pseudorotunda; a pneumatic quadrate; a foramen aereum in the lower jaw; periotic pneumatic cavities in the dorsal, central and rostral positions; a quadrate cotylus at the anterior base of the parocciput; a bipartite quadrate articulation with dermal and endochondral bones—anteriorly with the prootic, squamosal and laterosphenoid, posteriorly with the prootic and otocapital; a squamosal shelf over the ear region; antero-medial origin of the temporal musculature; two pneumatic cavities surrounding the cerebral carotid; unserrated teeth with a constricted neck⁷; bony tooth roots with an enclosed, oval resorption pit⁷. None of these features is known in theropod dinosaurs. The 'lateral depression' of *Saurornithoides* has no similarity to the periotic sinuses.

On the basis of present evidence, we feel that an argument of close relationship to crocodilians is the most parsimonious hypothesis available for the ancestry of birds.

K. N. WHETSTONE
L. D. MARTIN

Department of Systematics
and Ecology,
Museum of Natural History,
University of Kansas,
Lawrence, Kansas 66045

1. Baird, I. L. in *Biology of the Reptilia: Morphology B*. Vol. 2 (eds Gans, C. & Parsons, T. S.) 193-275 (Academic, London, 1970).
2. Whetstone, K. N. & Martin, L. D. *Nature* **279**, 234-236 (1979).
3. Osmólska, H., Roniewicz, E. & Barsbold, R. *Palaeont. pol.* **27**, 103-143 (1972).
4. Colbert, E. H. & Russell, D. A. *Am. Mus. Nov.* No. 2380, 1-49 (1969).
5. Ostrom, J. H. *Nature* **242**, 136 (1973).
6. Walker, A. D. *Nature* **237**, 257-263 (1972).
7. Martin, L. D., Stewart, J. D. & Whetstone, K. N. *Auk* **97**, 86-93 (1980).

BOOK REVIEWS

History and non-history

Dorothy Hodgkin

Sage: A Life of J. D. Bernal. By Maurice Goldsmith. Pp.288. (Hutchinson: 1980.) £8.95.

WHEN he spoke in memory of J. D. Bernal in January, 1972, Lord Snow said "I strongly advise that his biography should not be written until the end of the coming decade . . . there is so much in this rich life to learn about and to interpret". The decade is coming to an end, a large amount of material connected with Bernal's scientific and other work and some personal papers have been sorted out, and a catalogue — nearly ready — is being prepared in the archives of Cambridge University library. So, on Lord Snow's prescription, a serious book on Bernal could now be written; and, perhaps, the sooner the better, while there are still many living who knew him.

Maurice Goldsmith's book is not the book that is needed. It is not even a good forerunner of that book. It is too confused and inaccurate. Goldsmith does not understand Bernal well enough as a person, what he cared for and particularly his scientific work and achievement. This Bernal himself felt, when, semi-paralysed, he was visited by Goldsmith and learned that he wanted to write his biography. Bernal asked his wife to discourage Goldsmith from writing, which she did as far as she could, arranging for an alternative biographical study to be made and refusing Goldsmith access to the Cambridge archives. Knowing something of the state of the archives in the past few years I have every sympathy with her. Knowledge of its contents would not have removed the major defects of this book.

The book sets out to cover Bernal's life and work in 14 chapters, from his childhood in Ireland, where he was born in 1901, to his death in London in 1971. The early part is largely taken from autobiographical sketches written by Bernal, much of the rest from conversations with friends and colleagues, from newspaper reports of the time and some from Bernal's own writings. So there are many stories which bring back vivid memories of him, of his early passion for science and experiments at home, at school and Cambridge, of Sir William Bragg and the Royal Institution, and the beginning of the X-ray analysis of crystals,

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

J. D. Bernal at the inauguration of a new building of the Institute for Structure Research in Germany, March 1962. Dorothy Hodgkin is in the background.

of Max Perutz coming to work with him at Cambridge, of his war work on air-raid precautions and with Lord Mountbatten. It was at Cambridge that Bernal acquired the nickname Sage which many of his early friends used and which gives the book its title. He read widely and to his undergraduate friends seemed extraordinarily learned. Goldsmith himself knew him best at a later stage of his life, through their common interest in the use of science, science policy, the social function of science and the science of the sciences; these are subjects dealt with at some length. There are also chapters on Bernal's relations with the Soviet Union, on the development of international organizations for science and for peace after the War, on the political controversies that troubled both his early and later career, and on his work on science in history.

Unfortunately the text is scattered with inaccuracies — in most cases easily avoidable — confusions and misconceptions. For example, it is recorded that Desmond Bernal had one brother and two sisters; he had, in fact, two brothers, Kevin and Godfrey, who are still alive; one of his two sisters died in infancy. Later the text runs: "In June 1945, Bernal was in Moscow for the 220th anniversary of the Academy of Sciences. His friend Frédéric Joliot-Curie was also there . . . together with Joliot, Bernal began discussions with a few Soviet scientists on a

proposed new international body". Actually Bernal was prevented from going to the Moscow celebrations by the British government — later it is said, quite correctly, that the visit he made to Moscow in 1949 was the first since 1934. Then, about the long paper by Bernal and Fankuchen on tobacco mosaic virus: "It was most fortunate that Fankuchen finally wrote up that important paper, because Bernal would never have done so". This is invention. Anyone who knew them both would know only Bernal could have written the major part of that paper. Fankuchen was an excellent experimentalist, but his role at the end was to add a few exact figures and get the paper accepted for publication.

From childhood Bernal was passionately interested in science, in carrying out experiments himself, thinking about his own finds and those of others. As the dustcover says, "although primarily a crystallographer, he produced and provoked ideas over almost the entire range of scientific enquiries". Yet only one chapter of the 14 is devoted to the main body of his scientific work and this is extraordinarily confused and full of doubtful comments. What was really involved in his first crystallographic measurements on the sterols, and how these were linked with the work on oestrin and affected the general stream of chemical research, is never clear. Nor is the

Dorothy Hodgkin is Emeritus Professor at the University of Oxford, and a Fellow of Somerville College and Wolfson College, Oxford.

significance of his discovery of the conditions under which proteins and viruses could give detailed X-ray diffraction effects, work which opened the way to the elucidation of their structures and to much more, the solution of the structure of DNA and the understanding of molecular biology as we know it now. I read again the review Bernal wrote in 1968 of Watson's book, *The Double Helix*, and did not find it embarrassing as Goldsmith suggests. Bernal says specifically: "My name does not appear, and rightly, in the double helix story". That he should feel, all the same, that he had so many clues that he should have been able to see the solution himself was only natural — Pauling felt just the same on reading the book; and Bernal had an added reason in that he had himself taken X-ray photographs of nucleic acids as long ago as 1937. Incidentally, the remark that Furberg established the structure of DNA is so wrong it must be a misprint.

Other Goldsmith comments: the implication of the sentence "his own major, purely scientific contributions were made before 1939" that Bernal's work was either not his own or not major — is surely wrong. After the war Bernal set up a

number of very good research groups at Birkbeck, which he left much to themselves — he strongly believed, from his early work with Sir William Bragg, that independence was a good thing — he himself working mainly on the liquid state; it was from this research that his 1959 paper on the structure of liquids emerged, arguably the most brilliant he ever wrote. Another serious misunderstanding: "he had a hurting yet non-deliberate blindness about key work done by members of his staff". Characteristically, Bernal was the most generous of colleagues, generous with ideas, help and credit (as indeed is said elsewhere). Any blindness of the kind mentioned was extremely rare with him — as with children in many large families, his scientific children sometimes felt they had less than they would have liked of their parents' attention. To most he seemed kind beyond their deserts.

Many of the failings of Goldsmith's treatment of his scientific work apply to other sections; his judgement seems to me often very wrong. He relies too much on casual, sometimes malicious, gossip. John Desmond Bernal's life and work deserves a very much more serious study and appraisal. □

More *Drosophila*

Peter Lawrence

The Genetics and Biology of Drosophila, Vol.2d. Edited by M. Ashburner and T.R.F. Wright. Pp.702. (Academic: 1980.) £54, \$124.50.

THIS volume is the last in the series dealing with the genetics and biology of the individual fruit fly. Volume 3, which is in preparation, will take us away into the biology of populations, taxonomy, ecology and evolution.

I have already taken the opportunity this multi-authored work has given me to malign multi-authored works in general (*Nature* 283, 116; 1980). One criticism of the earlier volumes was the way topics are jumbled up, which makes it necessary for the specialist to buy several volumes when he may only want to read a chapter or two in each. Another was that there appeared to be a lack of discipline which led to chapters being over-long and containing evanescent material, making the individual volumes unnecessarily bulky and expensive. Although I think Vol.2d is a legitimate target for the same attacks, it contains several good chapters which cover related topics and is therefore easier to defend.

This volume covers lampbrush chromosomes, sex determination, dosage compensation, oogenesis and spermatogenesis. There are chapters on the structure and

development of the epidermis, nervous system and tracheal system and three chapters which appear eccentric in this company — on neuromuscular physiology, fly pigments, and even one on viruses.

Most chapters are strong, concentrating in the main on useful facts rather than idle discussion. There are many good things, for instance tables of enzyme activities in flies bearing different X-autosome ratios, pictures and drawings of the central nervous system and new illustrations of the larval cuticular sensilla, tables referring the reader to papers on the structure of the imaginal discs, discussion of the types of cell junctions found in epithelia, drawings of the ramifications of the tracheal system, and a very useful compendium of the effects of eye colour mutants. Examples of idle discussion of the abstruse are much of the chapters on sex determination and viruses, and the sections on the rate of oogenesis in umpteen species of *Drosophila* and on cell lineage in ommatidial development. This volume, like bits of its predecessors, will be a valuable resource. Editing huge books like this is no delight, so please raise your glasses to Ashburner and Wright! □

Peter Lawrence is on the staff of the MRC Laboratory of Molecular Biology, Cambridge, UK.

Gut reactions

G.J. Dockray

Gastrointestinal Hormones. Comprehensive Endocrinology. Edited by George B. Jerzy Glass. Pp.1008. (Raven: 1980.) \$85, \$115.60 outside the USA.

TEN years ago secretin, gastrin and cholecystokinin were the only hormonal peptides to have been isolated from the gut and characterized. Today we know the structure of over a dozen distinct biologically active gut peptides. The function of most of these is still uncertain. However, it seems likely that while some function as true hormones, others may act locally by diffusion to nearby cells (paracrine effects), and still others are secreted by nerves and have neuro-regulatory roles. The editor of this book has rightly taken a broad view of gastrointestinal hormones, and has included chapters on both the classical hormones (secretin, gastrin, cholecystokinin) and a wide range of other active peptides.

Gut endocrinologists have not been reticent about reviewing the rapid advances in their field, and at least five other books (generally conference proceedings) devoted to the gut endocrine system have appeared in recent years. The value of the present book lies in its comprehensive approach to the subject, and in this it lives up to the title of the series.

There are 42 chapters contributed by workers from 33 different laboratories, all active in this area. The division of the chapters into sections has been rather arbitrary and the greater part of the book is essentially a systematic coverage of the localization, isolation, characterization, biological properties, receptor binding, physiology and measurement of gut peptides. A final series of chapters deals with those "candidate" hormones which have not been isolated but whose existence is inferred from physiological studies, and also with new peptides which are in the process of being isolated from hog intestine by Viktor Mutt and his collaborators.

In the not too distant future it seems probable that we will know of 20 or 30 chemically defined and biologically distinct peptides which may control digestion. This field is obviously moving quickly, and it is therefore welcome to see that the publishers have managed to produce the book quite rapidly (some references are barely a year old). Although it is inevitable that much of the book will soon be out of date, it is still essential reading for anyone who wants to keep up with advances in this area. It will also provide an invaluable source for understanding developments not just in gut endocrinology, but also in the study of peptides in central and peripheral nerves. □

G.J. Dockray is Reader in Physiology at the University of Liverpool.

Diagenesis by numbers

R.G.C. Bathurst

Early Diagenesis: A Theoretical Approach. By Robert A. Berner. Pp.241. (Princeton University Press: 1980.) Hardback \$25, £13.70; paperback \$9.50, £5.25.

THIS most readable and helpful book should be welcomed by all who wish to understand chemical processes in diagenesis, be they students, teachers or research workers. Berner's approach is rigorously logical: he selects various problems in early diagenesis, shows how these may be clarified and simplified, and how to pose sensible questions about them, and, finally, indicates how to derive models that are both testable quantitatively and valuable as guides to prediction. His mode of thought combines a rigorous quantitative approach (calculus at the level of simple integration) with a careful appreciation of such seemingly unquantifiable processes as bioturbation and bacterial decay. Above all, he is concerned with the rates at which different processes work. In this book he deals only with diagenesis in the first 100m or so of burial, thus avoiding complicating factors such as higher temperatures, uplift, weathering and metamorphism.

In Part I there are five chapters devoted to theory. After a short introduction, he presents a quantitative approach to basic concepts such as rates of burial, steady-state diagenesis and diffusive and advective fluxes. Next he examines in detail the quantitative expression of compaction, rate of deposition, rate of flow of water across a sediment-water interface, molecular diffusion and the application of Fick's laws, and how to accommodate bioturbation into a quantitative model. The following two chapters cover various chemical processes that are the driving forces for diagenetic change. These include adsorption, ion exchange, radioactive decay, microbial metabolic reactions, the energetics of precipitation, nucleation, crystal growth, transport control and dissolution, and the general matter of diagenetic redistribution, with a section on causative factors in authigenic processes.

Part II is about applications and in it Berner discusses and enlarges upon work that has been carried out in real situations. He starts with a consideration of marine sediments of the continental margins; topics include diagenesis within the zone of bioturbation, relying on work done in Long Island Sound, touching upon such matters as irrigation and molecular diffusion of ammonia into burrows constantly flushed with oxygenated water, bacterial sulphate reduction, ammonia formation, phosphate diagenesis and methane formation. He then comes to pelagic (deep-sea) sediments, dealing with carbonate dissolution and the lysocline and

compensation depth, burial flux and compaction, the dissolution of opaline silica, the diagenesis of suboxic organic matter and of radioisotopes, and volcanic-seawater reactions. The final chapter embraces non-marine sediments (fresh to brackish water) and treats salinity fluctuations, iron and manganese diagenesis and hypersaline sediments. There is a 13-page bibliography and an efficient index. The chapters each have running headlines and these, combined with clear paragraph

headings, make it easy for the reader to find his way around.

Berner's writing is superbly clear and readers should find the book both interesting and stimulating. It excels as an exercise in controlled scientific imagination and sets a high standard for future studies of recent diagenesis. □

R.G.C. Bathurst is Professor of Geology at the University of Liverpool and of Petroleum Geology (II) at the University of Bergen.

Attractions of magnetic interactions

B. R. Coles

Interactions in Magnetically Ordered Solids. By K. P. Sinha and N. Kumar. Pp.180. (Oxford University Press: 1980.) £12.50, \$36.50.

THIS short book covers a lot of ground, although the authors explain at the outset that they have restricted themselves to localized moments in insulators and semiconductors. They reveal some ambivalence, however, towards conduction electrons, recognizing their importance as mediators of magnetic interactions (albeit in a rather concentrated treatment in which the sudden appearance of the word "impurity" may confuse many readers) but rather oddly entitling their last chapter "Conduction electron magnon interaction in ferromagnetic insulators" — a contradiction in terms not clarified by a brief final paragraph on spin-disorder resistivity which introduces de Gennes and Friedel's treatment of magnetic metals.

The problem of over-compression of the subject matter runs right through the book, perhaps the most glaring example being a section of one and a half pages of text and three figures which is introduced as "a brief survey of the thermodynamic properties of magnetic insulators".

As a result, it is difficult to see for whom the book is intended. Any given topic is dealt with so briefly as to constitute little more than a signpost to an area of interest, any real understanding of which by a student will require extensive reading elsewhere. Furthermore, the guidance to such reading is often very limited. For example, no reference to the book of Marshall and Lovesey (from the same publishers) is given in the short chapter on neutron-magnon interactions, which devotes two pages to magnon dispersion relations and nothing (in spite of the section introduction's claim) to magnon lifetimes. Perhaps the more theoretically inclined graduate student will use this book

to pick up hints about arenas in which he can flex his mathematical muscles without having to acquire much feeling for the nature of the materials in which the fascinating phenomena of magnetism manifest themselves. □

B. R. Coles is Professor of Solid State Physics at Imperial College, University of London.

Art of catalysis

D.E.W. Vaughan

Heterogeneous Catalysis in Practice. By C.N. Satterfield. Pp.416. (McGraw-Hill: 1980.) \$36.30, £16.15.

IN this book Professor Satterfield has squeezed into less than 400 pages the essential characteristics of catalysis and many of the major industrial catalytic processes. He has not attempted to be either up to date (there are few references later than 1976), or comprehensive in his coverage, but instead presents overviews that give the reader an appreciation for the processes and the associated practical problems ever present in "real" systems. The "art" side of catalysis is given a fairly complete coverage. Throughout, the references are secondary rather than primary; for example, few patents are cited. Overall, the treatment is directed toward the chemical engineer rather than the chemist.

The first half of the book deals with the necessary physical-inorganic chemistry of catalysis and catalysts, with chapters on sorption, kinetics, catalyst preparation and characterization. These are brief, and often only define the terms, nomenclature and methods of catalytic chemistry. The reader is supplied with appropriate

references if a deeper understanding is desired, both in the form of chapter bibliographies and a general literature overview at the end of the book. Throughout, the emphasis is on practicality, real problems and working systems. The chapter on catalyst preparation and manufacture (avoided in most books) is a reminder of the multitudinous problems that can be built into a catalyst before measurements or tests are made on it — a sobering reminder that a knowledge of the preparatory details is often essential to a complete interpretation of catalyst evaluation data.

The second part of the book surveys areas of industrially important catalytic chemistry, with chapters on supported metal catalysts, acid and zeolite catalysts, catalytic oxidation, processing of petroleum hydrocarbons, and synthesis gas and associated processes. These chapters trace the development of process catalysts up to present practice, focussing on details of catalyst composition and process conditions. The chapter on synthesis gas is particularly timely in view of the renewed interest in CO and H₂ as sources of

synthetic chemicals, and includes discussions of steam reforming, Fischer-Tropsch synthesis, water-gas shift reactions, methanation, methanol synthesis and ammonia synthesis. Details are generally accurate.

The final chapter on experimental methods covers the engineering details of reactor design, heat and mass transfer, and various types of reactors useful in the laboratory. This is useful to the chemist in that it is reminder of many important non-catalyst effects on experimental results.

The book is eminently readable, in the sense that the author gets to the point in clear prose, and moves on to the next subject. In addition to use in courses in catalysis, the book will provide a valuable review of the subject for the experienced worker in the field, in that its broad coverage of practical catalyst systems makes it a good initial reference for catalysts and process conditions. □

D.E.W. Vaughan is a Research Associate with the Exxon Research and Engineering Company, working in the areas of sorbent and catalytic materials.

Interactions of binary stars

R. J. Tayler

Close Binary Stars: Observation and Interpretation. Edited by M. J. Plavec and R. K. Ulrich. Pp.598. (Reidel: 1980.) Hardback Dfl.130, \$68.50; paperback Dfl.60, \$31.50.

BINARY stars have always been important for astronomers because the only direct way of determining stellar masses involves the application of Kepler's laws to the observed motions of the components. In addition, a study of eclipsing binaries can give information about stellar radii and variation of brightness across a stellar disk. It has, however, become clear only fairly recently that close binary stars are involved in some of the most violent and exotic phenomena in the Universe.

These phenomena are associated with mass transfer between the components. Most stars increase in size during their life history and a member of a close binary may become so large that its surface layers are attracted to the companion star; this is called Roche lobe overflow. In addition, some stars lose mass continuously and some of this may be captured by the companion. This exchange can have important effects on the evolution of the system. If the initially more massive star becomes the less massive, an ill-matched binary can result in which it appears that the lower mass star has evolved more rapidly than the more massive, in contradiction to basic ideas about stellar evolution. The most dramatic effects occur when one of the component stars is a highly compact star, a white dwarf, a neutron star

or a black hole, when a very large release of gravitational energy accompanies mass transfer. This process is important in many X-ray sources, novae and cataclysmic variables.

This book is the proceedings of a symposium held in Toronto in the summer of 1979. It contains over a hundred contributions and there are few papers which contain as many as ten pages. As a result, the non-specialist may have some difficulty in discovering what are the key questions and which are the most important papers. I am sorry that there is not a small number of lengthy reviews, since it is clear that there are some important unsolved problems which merit fuller discussion than they receive here. For example, the manner in which close binary systems are formed is not clear and there are some particularly difficult problems and sharp controversies relating to the structure of binaries that are so close that they really form a single star with two nuclei. Further, most calculations of stellar evolution involving mass transfer have assumed conservation of mass and angular momentum by the system, whereas an uncertain amount of each will escape.

Although close binaries are of wide general interest to astronomers, this book is most suitable for those who are already working in the subject. □

R. J. Tayler is Professor of Astronomy at the University of Sussex, author of three elementary astronomy textbooks and Managing Editor of Monthly Notices of the Royal Astronomical Society.

Andean archaeology

John Coles

Guitarrero Cave: Early Man in the Andes. Edited by Thomas F. Lynch. Pp.328. (Academic: 1980.) \$20, £13.60.

FOR some years now it has been an archaeological conceit to speak of interdisciplinary projects as the sole way of comprehending any ancient site, settlement or landscape. In few cases have these concepts been achieved, and instead the archaeological world has been presented with rather feeble attempts to coordinate and integrate the variety of natural scientific techniques that can be brought to bear upon most archaeological sites or areas. In the present book we have just such an effort, one which succeeds in part — but not wholly — in demonstrating the virtues of an interdisciplinary approach.

The cave of Guitarrero lies high (2580m) in an intermontane valley bounded by the Cordillera Blanca and C. Negra in Peru. The archaeologist will see the possibilities for an ecological research project, with dramatic seasonal variations in environmental conditions presumably being welcomed by the ancient inhabitants, and indeed this form of project is what was conceived and executed. The site was excavated, and its stratigraphical deposits dated by radiocarbon to a period which on balance seems to fall between 10,000 BP and 7,000 BP. The environmental implications and attempted reconstruction of the ancient landscapes are heavily reliant on pollen analyses, and there is relatively little on geomorphological processes which one could conceive of as affecting the landscape to a significant degree. Nonetheless, this initial section of the book sets the scene for the major thrust of the project, the study of the quite extraordinarily abundant remains of plants. This is explored through exhaustive analysis of maize and beans, with essays on the origin and early development of cultivation in highland Peru.

A third section provides a full description of the multifarious stone artifacts from the major stratigraphic phases of the cave occupation, and is somewhat tedious in approach. More interesting by virtue of rarity are the bone and wood artifacts, and remains of cordage, basketry and textiles which conditions in parts of the cave have allowed to survive. The final summary and overview of the site emphasizes this organic content which alone would have made the excavation worthwhile.

Yet, as regards disciplines, multidisciplinary it is, interdisciplinary it is not. Archaeologists may never be able to achieve the latter if they remain content with the former. □

John Coles is Professor of European Prehistory at the University of Cambridge.

BOOKS RECEIVED

Mathematics

- BOWEN, B.D. and WEISBERG, H.F. *An Introduction to Data Analysis*. Pp.213. Hbk ISBN 0-7167-1173-7; pbk ISBN 0-7167-1174-5. (W.H. Freeman: 1980.) Hbk £8.80; pbk £3.95.
- ELANDT-JOHNSON, R. and JOHNSON, N. L. *Survival Models and Data Analysis*. Pp.457. ISBN 0-471-03174-7. (Wiley: 1980.) £18.70.
- TJUR, T. *Probability Based on Radon Measures*. Pp.232. ISBN 0-471-27824-6. (Wiley: 1980.) £18.50.

Physics

- AGA-JANIAN, A.H. (Compiled by.) *Mosfet Technologies: A Comprehensive Bibliography*. Pp.377. ISBN 0-306-65193-9. (Plenum: 1980.) \$95.
- DALTON, B.J. *et al.* (eds). *Theory and Applications of Moment Methods in Many-Fermion Systems*. Proceedings of the International Conference held at Iowa State University, Ames, Iowa, September 1979. Pp.511. ISBN 0-306-40463-X. (Plenum: 1980.) \$59.50.
- DUFFIN, W.J. *Electricity and Magnetism*. 3rd Edn. Pp.467. Flexi ISBN 0-07-084111-X. (McGraw-Hill: 1980.) £7.50.
- KANE, J. W. and STERNHEIM, M. M. *Physics*. S.I. Version. Pp.664. ISBN 0-471-08036. (Wiley: 1980.) £13.80.
- LANCASTER, G. *DC and AC Circuits*. 2nd Edn. Pp.325. Hbk ISBN 0-19-85148-X; pbk ISBN 0-19-851849-8. (Oxford University Press: 1980.) Hbk £20; pbk £9.95.
- MARTON, L. and MARTON, C. (eds). *Advances in Electronics and Electron Physics*, Vol.54. Pp.318. ISBN 0-12-014654-1. (Academic: 1980.) \$38.50.
- PREPARATA, G. and AUBERT, J.-J. (eds). *Probing Hadrons with Leptons*. Proceedings of the 4th Seminar held in Erice, Sicily, March 1979. Pp.507. ISBN 0-306-40438-9. (Plenum: 1980.) \$59.50.
- RICHARD, P. (ed.) *Atomic Physics Accelerators*. Vol.17, *Methods of Experimental Physics*. Pp.641. ISBN 0-12-475959-9. (Academic: 1980.) \$70.
- SEPTIER, A. (ed.) *Applied Charged Particle Optics*. Part A. *Advances in Electronics and Electron Physics Supplement* 13. Pp.356. ISBN 0-12-014573-1. (Academic: 1980.) \$41.
- TURCHI, P. J. (ed.) *Megagauss Physics and Technology*. Proceedings of the 2nd International Conference held in Washington D.C., May-June 1979. Pp.683. ISBN 0-306-40461-3. (Plenum: 1980.) \$69.50.

Chemistry

- CRIDDLE, W. J. and ELLIS, G. P. *Spectral and Chemical Characterization of Compounds. A Laboratory Handbook*. 2nd Edn. Pp. 115. Hbk ISBN 0-471-27813-0; pbk ISBN 0-471-27812-2. (Wiley: 1980.) Hbk £13, \$38.50; pbk £4.50.
- FRANZOSINI, P. and SANESI, M. (eds). *Thermodynamic and Transport Properties of Organic Salts*. IUPAC Chemical Data Series, No. 28. Pp.376. ISBN 0-08-022378-8. (Pergamon: 1980.) \$90.
- KUWANA, T. (ed.) *Physical Methods in Modern Chemical Analysis* Vol.2. Pp. 411. ISBN 0-12-430802-3. (Academic: 1980.) \$45.
- ROSENBLATT, G. M. and WORRELL, W. L. (eds). *Progress in Solid State Chemistry*, Vol.12. 332. ISBN 0-08-022846-1. (Pergamon: 1980.) \$34.
- SAMMES, P. G. (ed.). *Topics in Antibiotic Chemistry*, Vol.5. Pp.312. ISBN 0-85312-191-5. (Ellis Horwood Limited: 1980.) Distributed by Wiley. £25.
- SVEHLA, G. (ed.) *Wilson and Wilson's Comprehensive Analytical Chemistry*, Vol.10. *Organic Spot Test Analysis: The History of Analytical Chemistry*. Pp.282. ISBN 0-444-41859-8. (Elsevier Scientific: 1980.) \$73.25, Dfl. 73.25.
- VOL'PIN, M. E. (ed.). *Chemistry Reviews*, Vol.2 (1980). Soviet Scientific Reviews, Section B. Pp.469. ISBN 3-7186-0018-8. (Harwood: 1980.) \$90.

Technology

- COUNCIL ON ENVIRONMENTAL QUALITY AND THE DEPARTMENT OF STATE. BARBEY, G. O. (Study Director). *The Global 2000 Report to the President of the U.S. entering the 21st Century*. Vol.II: *The Technical Report*. Pp.766. ISBN 0-08-024618-4. (Pergamon: 1980.) £20.
- DOLMAN, A. J. (ed.). *Global Planning and Resource Management. Towards International Decision Making in a Divided World*. Pp.262. Hbk ISBN 0-08-026309-7; pbk ISBN 0-08-026309-8. (Pergamon: 1980.) Hbk np.; pbk £3.50.
- FRANCIS, W. and PETERS, M.C. *Fuels and Fuel Technology. A Summarized Manual*. 2nd (SI) Edn. Pp.716. Hbk ISBN 0-08-025249; flexi ISBN 0-08-025250-8. (Pergamon: 1980.) Hbk £42.50, \$95; flexi £14.95, \$34.50.
- HASSELMAN, D.P.H. and HELLER, R.A. *Thermal Stresses in Severe Environments*. Proceedings of the International Conference held at Virginia Polytechnic Institute and State University, Blacksburg, March 1980. Pp.737. ISBN 0-306-40544-X. (Plenum: 1980.) \$75.
- HERTZBERG, R.W. and MANSON, J.A. *Fatigue of Engineering Plastics*. Pp.295. ISBN 0-12-343550-1. (Academic: 1980.) \$35.50.
- THE INSTITUTE OF CERAMICS. *Health & Safety in Ceramics: A Guide for Educational Workshops & Studios*. Pp.36. Flexi ISBN 0-08-026173-6. (Pergamon: 1980.) £2.
- NORTHROP, C. J. M. Jr (ed.). *Scientific Basis for Nuclear Waste Management*, Vol.2. Proceedings of the International Symposium, sponsored by the Materials Research Society, held in Boston, Massachusetts, November 1979. Pp.936. ISBN 0-306-40550-4. (Plenum: 1980.) \$65.

WILSON, R. (ed.). *Energy for the Year 2000*. Pp.401. ISBN 0-306-40540-7. (Plenum: 1980.) \$42.50.

ZALESKI, E. and WIENHART, H. *Technology Transfer between East and West*. Pp.435. Flexi ISBN 92-64-12125-0. (OECD: Château de la Muette, 2, rue André-Pascal, 75775 Cedex 16, 1980.) Np.

Earth Sciences

- CARP, E. (compiled by) *Directory of Wetlands of International Importance in the Western Palearctic*. Pp.506. Flexi ISBN 2-88032-300-2. (UN Environment Programme and the International Union for Conservation of Nature and Natural Resources: 1980.) \$27.50 + postage, from Unipub, 345 Park Avenue South, New York 10010, USA, and Bowker Publishing Company, P.O. Box 5, Epping, Essex CM16 4BU.
- COPE, J.C.W. *et al.* *A Correlation of Jurassic Rocks in the British Isles*. Geological Society Special Report No. 14, Part 1. Pp.74. Flexi ISBN 0-632-00712-5. (Blackwell Scientific: 1980.) £8.
- FROST, D.V. and SMART, J.G.O. *Geology of the Country North of Derby*. Memoir for 1:50,000 Geological Sheet 125. Geological Survey of Great Britain: England and Wales. Pp.200. ISBN 0-11-8841-19-X. (HMSO: 1979.) £18.
- KRAFFT, D. and DRAFFT, M. *Volcanoes: Earth's Awakening*. Pp.160. ISBN 0-8437-3760-3, ISBN 0-8437-3761 (deluxe). (Hammond Incorporated, Maplewood, New Jersey 07040: 1980.) Np.
- MERRILL, R. B. (ed.). *Proceedings of the Conference on The Lunar Highlands Crust*, Houston, Texas, November 1979. *Gosmochimica Acta*, Supplement 12. Pp.505. ISBN 0-08-026304-6. (Pergamon: 1980.) \$52.
- RUSSELL, P. J. (ed.). *Aquiline Thesaurus*. Pp. 684. ISBN 0-85312-266-0. (Ellis Horwood Limited: 1980.) £45.
- TURNER, F. J. *Metamorphic Petrology. Mineralogical, Field, and Tectonic Aspects*. 2nd Edn. Pp.524. ISBN 0-07-065501-4. (Hemisphere Publishing: 1980.) £28.50.
- YEN, T. F. and WALSH, D. (eds). *Energy and Resource Development of Continental Margins*. Pp.238. ISBN 0-08-025127. (Pergamon: 1980.) £15.

Biological Sciences

- BACHMANN, P.A. *Leukaemias, Lymphomas and Papillomas: Comparative Aspects*. Munich Symposia on Microbiology. Pp.273. Flexi ISBN 0-85066-213-3. (Taylor & Francis: 1980.) £9.50.
- BRISTOW, A. *The Sex Life of Plants: A Study of the Secrets of Reproduction*. Pp.223. Pbk ISBN 0-450-04667-2. (New English Library Limited: Barnard's Inn, Holborn, London, EC1N 2JR, 1980.) £1.25.
- DUPONT, H. L. and PICKERING, L. K. *Infections of the Gastrointestinal Tract. Microbiology, Pathophysiology, and Clinical Features*. Pp.273. ISBN 0-306-40409-5. (Plenum Medical: 1980.) \$24.50.
- GENEROSO, W. M., SHELBY, M. D. and de SERRES, F. J. (eds). *DNA Repair and Mutagenesis in Eukaryotes*. Basic Life Sciences, Vol.15. Proceedings of the Symposium sponsored by the National Institute of Environmental Health Sciences, held in Atlanta, Georgia, June 1979. Pp.458. ISBN 0-306-40552-0. (Plenum: 1980.) \$49.50.
- GILLOTT, C. *Entomology*. Pp.729. Flexi ISBN 0-306-40514-8. (Plenum: 1980.) \$22.50.
- HECHT, M.K., STEERE, W.C. and WALLACE, B. (eds). *Evolutionary Biology*, Vol.13. Pp.301. ISBN 0-306-40510-5. (Plenum: 1980.) \$32.50.
- KARREMAN, G. (ed.). *Co-operative Phenomena in Biology*. Pp.246. ISBN 0-08-023186-1. (Pergamon: 1980.) £21.25.
- MAKI, A.W., DICKSON, K.L. and CAIRNS, J.C. Jr (eds). *Biotransformation and the Fate of Chemicals in the Aquatic Environment*. Proceedings of a Workshop held at the University of Michigan Biological Station, Pellston, Michigan, August 1979. Pp.150. ISBN 0-914826-28-X. (American Society for Microbiology: Washington DC, 1980.) \$12.
- MANI, M.S. and GIDDINGS, L.E. *Ecology of Highlands*. Monographiae Biologicae, Vol.40. Pp.236. ISBN 90-6193-093-6. (W. Junk: The Hague, 1980.) Dfl.110, \$58.
- RHOADS, D. C. and LUTZ, R. A. *Skeletal Growth of Aquatic Organisms: Biological Records of Environmental Change*. Topics in Geobiology, Vol.1. Pp.750. ISBN 0-306-40259-9. (Plenum: 1980.) \$47.50.
- ROODYN, D.B. *Subcellular Biochemistry*, Vol.7. Pp.412. ISBN 0-306-40485-0. (Plenum: 1980.) \$49.50.
- SANDLER, S.G., NUSBACHER, J. and SCHANFIELD, M.S. (eds). *Immunobiology of the Erythrocyte*. The American Red Cross 11th Annual Scientific Symposium, Washington D.C., May 1979. Pp.360. ISBN 0-8451-0043-2. (Alan R. Liss: New York, 1980.) \$40, Dfl.120.
- SIDDIQI, O. *et al.* (eds). *Development and Neurobiology of Drosophila*. Basic Life Sciences, Vol.16. Proceedings of the International Conference on Development and Behaviour of *Drosophila melanogaster*, held at the Tata Institute of Fundamental Research, Bombay, December 1979. Pp.496. ISBN 0-306-40559-8. (Plenum: 1980.) \$49.50.
- SPATZ, M. *et al.* (eds). *Circulatory and Developmental Aspects of Brain Metabolism*. Proceedings of the 2nd International Symposium on the Pathophysiology of Cerebral Energy Metabolism, organized and sponsored by the Serbian Academy of Sciences and Art, held in Belgrade, Yugoslavia, September 1979. Pp.446. ISBN 0-306-40542-3. (Plenum: 1980.) \$49.50.

- STANBURY, P. and PHIPPS, G. Australia's Animals Discovered. Pp.120. ISBN 0-08-024796-2. (Pergamon: 1980.) £23.40, £9.75.
- STEVENS, D.A. (ed.). Coccidioidomycosis. A Text. Pp.279. ISBN 0-306-40410-9. (Plenum: 1980.) \$28.50.
- VON FABER H. and HAID, H. Endokrinologie. Biochemie und Physiologie der Hormone. Pp.174. Pbk ISBN 3-8001-2489-0. (Verlag Eugen Ulmer: Postfach 10 32 7000 Stuttgart 1, 1980.) DM 17.80.
- WESTWOOD, J.C.N. The Hazard From Dangerous Exotic Diseases. Pp.223. ISBN 0-333-28360-0. (Macmillan: London, 1980.) £17.50.
- WOOD, J.H. (ed.). Neurobiology of Cerebrospinal Fluid. Pp.768. ISBN 0-306-40369-2. (Plenum: 1980.) \$60.50.

Applied Biological Sciences

- ASSCHER, A. W. The Challenge of Urinary Tract Infections. Pp.209. ISBN 0-12-790220-1. (Academic: 1980.) £15, \$34.50.
- BRANDLY, C. A. and CORNELIUS, C. E. (eds). Advances in Veterinary Science and Comparative Medicine, Vol.24. Pp.325. ISBN 0-12-039224-0. (Academic: 1980.) \$39.50.
- BREWSTER, M. A. and NAITO, H. K. (eds). Nutritional Elements and Clinical Biochemistry. Proceedings of the 3rd Annual Meeting of the National Academy of Clinical Biochemistry, held in New Orleans, Louisiana, July 1979. Pp.463. ISBN 0-306-40569-5. (Plenum: 1980.) \$45.
- DAVID, G. and PRICE, W.S. (eds). Human Artificial Insemination and Semen Preservation. Proceedings of the International Symposium held in Paris, April 1979. Pp.639. ISBN 0-306-40547-4. (Plenum: 1980.) \$65.
- DAWKINS, M. S. Animal Suffering. The Science of Animal Welfare. Pp.150. Hbk ISBN 0-412-22580-8; pbk ISBN 0-412-22590-5. (Chapman & Hall: 1980.) Hbk £7.50; pbk £3.95.
- EYSENK, H.J. The Causes and Effects of Smoking. Pp.397. ISBN 0-05117-186-9. (Maurice Temple Smith: London, 1980.) £16.50.
- HARRIS, C. C., TRUMP, B. F. and STONER, G. D. (eds). Methods in Cell Biology. Prepared under the Auspices of the American Society of Cell Biology, Vol.21: Normal Human Tissue and Cell Culture, B. Endocrine, Urogenital, and Gastrointestinal Systems. Pp.512. ISBN 0-12-564140-0. (Academic: 1980.) \$49.50.
- HUME, D. A. and WEIDEMANN, M. J. Mitogenic Lymphocyte Transformation. A General Model for the Control of Mammalian Cell Proliferation and Differentiation. Research Monographs in Immunology, Vol. 2. Pp.251. ISBN 0-444-80218-3. (Elsevier/North-Holland Biomedical: 1980.) \$83, Dfl.170.
- IACOBELLI, S. *et al.* (eds). Progress in Cancer Research and Therapy, Vol.14. Hormones and Cancer. Pp.590. ISBN 0-89004-486-4. (Raven: 1980.) \$49.
- INGLIS, J. K. Introduction to Laboratory Animal Science and Technology. Pp.323. Hbk ISBN 0-08-023772-X; flexi ISBN 0-08-023771-1. (Pergamon: 1980.) Hbk £20; flexi £9.95.
- KOLBE, H. K. (ed.). Intra-uterine Devices Abstracts: A Guide to the Literature, 1978-1979. Population Information Library, Vol.1. Pp.569. ISBN 0-306-65191-2. (IFI/Plenum: 1980.) \$75.
- KOLBE, H. K. (ed.). Oral Contraceptives Abstracts. A Guide to the Literature, 1977-1979. Population Information Library, Vol.2. Pp. 563. ISBN 0-306-65192-0. (IFI/Plenum: 1980.) \$75.
- LEVY, A. *et al.* (eds). Endogenous Peptides and Centrally Acting Drugs. Progress in Biochemical Pharmacology, Vol.16. 24th Annual OHOL Biological Conference, Zichron Ya'acov, April 1979. Pp.160. ISBN 3-8055-0831-X. (Karger: Basel, 1980.) Sw.Fr. 82, DM 98, \$49.25.
- McLAREN, D.J. *Schistosoma mansoni*: The Parasite Surface in Relation to Host Immunity. Tropical Medicine Research Studies Series, 1. Pp.229. ISBN 0-471-27869-6. (Wiley: 1980.) £18.
- MURPHY, E. D. and BEAMER, W. G. (eds). Biology of Ovarian Neoplasia. A Series of Workshops on the Biology of Human Cancer, Report No.11. UICC Technical Report Series, Vol.50. Pp.132. Flexi ISBN 92-9018-050-1. (International Union Against Cancer: 3 rue du Conseil-Général, -1205 Geneva, Switzerland: 1980.) SwFr. 16.
- RIGTER, H. and CRABBE, J. C. (eds). Alcohol Tolerance and Dependence. Pp.455. ISBN 0-444-80212-6. (Elsevier/North-Holland Biomedical: 1980.) \$95, Dfl. 195.
- THOMAS, R.H. and PEREZ-MENDEZ, V. Advances in Radiation Protection and Dosimetry in Medicine. Proceedings of the Symposium held in Italy, September 1979. Pp.658. ISBN 0-306-40468-0. (Plenum: 1980.) \$69.50.
- WEETALL, H.H. and ROYER, G.P. (eds). Enzyme Engineering, Vol.5. Proceedings of the 5th International Conference, held in Henniker, New Hampshire, July 1979. Pp.485. ISBN 0-306-40471-0. (Plenum: 1980.) \$49.50.
- WINICK, M. (ed.). Nutrition and Gastroenterology. Current Concepts in Nutrition, Vol. 9. Pp.221. ISBN 0-471-08173-6. (Wiley: 1980.) £17.50.

Psychology

- LIBERMAN, I.P. *et al.* Handbook of Marital Therapy: A Positive Approach to Helping Troubled Relationships. Pp.262. ISBN 0-306-40235-1. (Plenum: 1980.) \$19.50.
- SINZ, R. Chronopsychophysiologie. Chronobiologie und Chronomedizin. Band 217. Pp.360. (Akademie-Verlag, DDR-108 Berlin: 1980.) DM 16.
- STADDON, F. E. R. (ed.). Limits to Action. The Allocation of Individual Behavior. Pp.308. ISBN 0-12-662650-2. (Academic: 1980.) £24.

Sociology

- BRIERLEY, J. Children's Well-Being. Pp.171. Pbk ISBN 0-85633-218-6. (NFER Publishing Company Ltd: Darville House, 2 Oxford Road East, Windsor, Berks SL1 2DQ, 1980.) £6.75.
- NEUBERGER, E. and TYSON, L. D'A. (eds). The Impact of International Economic Disturbances on the Soviet Union and Eastern Europe. Transmission and Response. Pp.493. ISBN 0-08-025102-1. (Pergamon: 1980.) £30.
- RA'ANAN, U. and ROCHE, J. P. *et al.* (eds). Ethnic Resurgence in Modern Democratic States: A Multidisciplinary Approach to Human Resources and Conflict. Pp.288. ISBN 0-08-024647-8. (Pergamon: 1980.) £15.
- STEWART, D. W. The Women's Movement in Community Politics in the U.S. The Role of Local Commissions on the Status of Women. Pp.146. ISBN 0-08-025971-5. (Pergamon: 1980.) £7.50.

History of Science

- CHANT, C. and FAUVEL, J. (eds). Darwin to Einstein. Pp.335. Hbk ISBN 0-582-49156-8; pbk ISBN 0-582-49157-6. (Longman: 1980.) Hbk £9.95; pbk £5.65.
- JACOBS, L.L. (ed.). Aspects of Vertebrate History. Essays in Honour of Edwin Harris Colbert. Pp.407. Hbk ISBN 0-89734-052-3; pbk ISBN 0-89734-053-1. (Museum of North Arizona Press: 1980.) Hbk \$22.50; pbk \$9.95.

Anthropology

- ROBSON, J. R. K. (ed.). Food, Ecology and Culture. Readings in the Anthropology of Dietary Practices. Pp.143. ISBN 0-677-16990-9. (Gordon & Breach: 1980.) \$27.50.
- SCHIFFER, M. B. (ed.). Advances in Archaeological Method and Theory, Vol.3. Pp.448. ISBN 0-12-003103-5. (Academic: 1980.) \$39.50.

General

- APPLE, A. Megasyntesis. Pp.512. Flexi ISBN 0-9690271-0-9. (Megasyntesis: P.O. Box 343, SubS. 11, University of Alberta, 1980.) \$10.
- AUDUBON, J.J. The Art of Audubon. The Complete Birds and Mammals. Pp.674. ISBN 0-354-04593-8. (Macdonald-Futura: 1980.) £15.95.
- BERGMANN, P.G. and DE SABBATA, V. (eds). Cosmology and Gravitation: Spin, Torsion, Rotation, and Supergravity. NATO Advanced Study Institutes Series, Vol.58. Proceedings of the NATO Advanced Study Institute held at the Ettore Majorana International Centre for Scientific Culture, Erice, Italy, May 1979. Pp.510. ISBN 0-306-40478-8. (Plenum: 1980.) \$65.
- DADANT, C.P. First Lessons in Beekeeping. Pp.127. ISBN 0-684-16747-6. (Schribner's: 1980.) \$7.95.
- DOBSON, F., HASSE, L. and DAVIS, R. Air-Sea Interaction. Pp.801. ISBN 0-306-40543-1. (Plenum: 1980.) \$39.50.
- GRANDJEAN, E. and VIGLIANI, E. (eds). Ergonomic Aspects of Visual Display Terminals. Proceedings of the International Workshop, Milan, March 1980. Pp.300. ISBN 0-85066-211-7. (Taylor & Francis: 1980.) £18.
- HULL, F. C. The Evaluation of Risk in Business Investment. Pp.177. Hbk ISBN 0-08-024075; flexi ISBN 0-08-024074-7. (Pergamon: 1980.) Hbk £12, \$27; flexi £6.50, \$15.
- LANGEWIESCHE, M. Venedig. Geschichte und Kunst. Erlebnis einer einzigartigen Stadt. Pp.284. Flexi ISBN 3-7701-0653-9. (DuMont Buchverlag Köln: 1979.) Np.
- LONG, F. A. and REPPY, F. (eds). The Genesis of New Weapons. Decision Making for Military R & D. Pp.210. ISBN 0-08-025973-1. (Pergamon: 1980.) £11.20.
- MANN, W.E. and HOFFMAN, E. The Man Who Dreamed of Tomorrow. A Conceptual Biography of Wilhelm Reich. Pp.295. ISBN 0-87477-143-9. (J.P. Tarcher, Inc: Los Angeles, 1980.) \$12.95.
- NEGANDHI, A. R. (ed.). Functioning of the Multinational Corporation: A Global Comparative Study. Pp.230. ISBN 0-08-025087-4. (Pergamon: 1980.) £9.
- PRUTHI, J. S. Spices and Condiments: Chemistry, Microbiology, Technology. Advances in Food Research Supplement, 4. Pp.449. ISBN 0-12-016464-7. (Academic: 1980.) \$39.50.
- ROXBURGH, N. Policy Responses to Resource Depletion: The Case of Mercury. Contemporary Studies in Economic and Financial Analysis, Vol.21. Pp.219. ISBN 0-89232-093-1. (Jai Press: Greenwich, Connecticut, 1980.) \$27.50.
- SAUNDERS, C. T. (ed.). East and West in the Energy Squeeze. Prospects for Co-operation. East-West European Economic Interaction Workshop Papers, 5. Pp.468. ISBN 0-333-30515-9. (Macmillan: 1980.) £20.
- STEVENSON, I. Cases of the Reincarnation Type, Vol.III. Twelve Cases in Lebanon and Turkey. Pp.384. ISBN 0-8139-0816-7. (The University Press of Virginia: 1980.) \$25.
- SURYANARAYANA, C. (ed.). Rapidly Quenched Metals: A Bibliography, 1973-1979. Pp. 278. ISBN 0-306-65194-7. (IFI/Plenum: 1980.) \$75.
- TURNER, J. Reckoning with the Beast. Animals, Pain, and Humanity in the Victorian Mind. Pp.190. ISBN 0-8018-2399-4. (The Johns Hopkins University Press: 1980.) \$14.

CORRESPONDENCE

Continued from p. 8

most of the students and younger zoological systematists (and now even some botanists) have adopted this strategy of dealing with the empirical world in the belief that they were coming to understand something new about the hierarchical structure of nature. Of course, we should have known all along that something was amiss since most of our older colleagues from an earlier and wiser generation had told us that the spread of cladistics will have an extremely deleterious effect on Mayrian (or Simpsonian, or Darwinian) taxonomy. Well, the damage is done: their taxonomy will never be the same again — wiped out, as it were, by an epidemic of cladistics brought on by thoughtless, unhygienic and scientifically irresponsible cladists.

Some of my colleagues doubtless (in the old sense) will consider it premature for me to write such congratulatory lines about Dr Halstead on the grounds that he knows almost nothing, and appears to be unfamiliar with the literature, of cladistics. But I remind them that with Halsteadian Truth, knowledge of cladistics is irrelevant for cladistics is a theoretical matter. So I say to Halstead that there can be no serious doubt (in the new sense, for I actually seem to be getting the hang of it!) about the importance of his discovery of false doubt to systematics and evolutionary biology. Application of Halsteadian Truth suddenly reveals why all those serious doubters have questioned the usefulness of gradualism as a doctrine. This is because, poor souls, they imagined that (1) the modern doctrine of gradualism as derived from population genetics has no known empirical relationship to the hierarchy of organisms, (2) that population genetics theory was designed to rescue Darwin's theory of natural selection when it faltered because of its untestability, and (3) that the applicability of gradualism as an explanation of taxic diversity is achieved by a simple extrapolation from artificial selection experiments and the changes in gene frequencies observed in nature. I am forced also to include among the serious doubters a variety of embryologists and developmental geneticists who, before the coming of Halsteadian Truth, were thought to be rather distinguished.

The innate wisdom Halstead shows about cladistics and evolutionary theory extends to his philosophy of science and politics. On the thinnest of pretexts he tries to convince us that his certain knowledge that evolution is gradual constitutes a perfect, and necessary, refutation of Marxist doctrine. Would Halstead, I wonder, imagine that the massive extinctions that have taken place in his gradualistic world also carry sinister implications for us? The class struggle? Or did Darwin only predict the end of his own theory? Halstead, this great seeker of truth, must really be rebuked for deliberately confounding science and politics and for attributing the troublesome notions of Social Darwinism to the entirely ethical and clear-thinking scientists and educators of the British Museum.

DONN E. ROSEN

Department of Ichthyology,
American Museum of Natural History,
New York

SIR — Beverly Halstead has drawn attention¹ to the ideological basis of the present exhibition policy at the Natural History Museum, and its implications. Other aspects of present policy are scarcely less disturbing. They relate to the display of the museum's collections and to the use of the building in which they are housed.

The museum's original exhibits were systematically arranged and a great number of genera and species were shown. The building in which they were housed was designed by Alfred Waterhouse (1830–1905), one of our greatest Victorian architects. It is a superb building and is listed as a Grade I Historic Building by the Department of the Environment. Waterhouse himself took great pains over the exterior and interior detailing and the whole building has an impressive unity.

The original exhibition galleries, whose plan and detailing reflected the order and hierarchy of nature, as then perceived, remained in use until the outbreak of war in 1939. The war led to closure of some galleries, and after the war nothing much happened for a long time. Museums generally were reacting against the old cases and galleries stuffed with objects, and the authorities of the British Museum (Natural History) did not seem to know what to do about the old systematic displays.

Eventually a policy seemed to emerge, exemplified by the new Fossil Mammal exhibit opened in 1972 in what was the old Fossil Mammal Gallery. In place of the dramatic long vista past mounted skeletons of fossil elephants, the continuity of the gallery was broken up and its original architectural character subdued, though some of the detail is still visible. Scientifically the new exhibit is much better than the old one, with a great deal of information on the animals and on the rocks from which they came, attractively displayed. As Halstead has pointed out², everyone, from a specialist to a child, can get something out of it.

So far, the museum had pursued the policy of showing a representative series from the collections which are its *raison d'être*. In this connection it is interesting that the British Museum Act 1963 makes no reference to the public exhibition of the collections. The explanations must be that in the eighteenth century, when the original acts were framed which the 1963 Act replaces, it was self-evident that a museum existed for this purpose.

Those who hoped that Fossil Mammals heralded the modernization of other galleries were soon to be disappointed. There was an abrupt change of policy. The museum's report³ for 1972–74 said that "Unhappily, the existing public exhibition does not match the museum's purpose . . . it has lagged far behind this century's revolution in the natural sciences. . . it fails to give a clear picture of natural history as the study of the world in which we live with principles which lie at the groundwork of our technologies, and which are the first principles of rational living". The failure seems disputable—the original exhibits largely gave such a picture, and the rest of the sentence is gibberish. (What are the first principles of rational living?)

The report outlined a new exhibition "much larger than the old" designed to "reflect all aspects of modern biology". It

gave four themes which were to be the basis for the new exhibition: Ecology; Life processes and behaviour; Evolution and diversity; and Man. The Hall of Human Biology (opened in 1977; actually a warren rather than a hall), Ecology (1978) and the new exhibits on Man and Dinosaurs criticized by Halstead, are the first fruits of the stated policy.

It is debatable to what extent the museum should set itself up to be the fountainhead of the fashionable parts of modern biological education. In the case of the new Dinosaur exhibit, the sequence of arguments presented in the exhibit is repeated almost word for word in the accompanying booklet. This exemplifies the pointlessness of the new exhibition policy, which employs a great deal of expensive equipment to put over concepts which are better expounded on paper, and which are but distantly related to the collections and function of the museum. There is no coherent relationship between the new exhibits, the collections and the building. The recent exhibits make minimal use of actual specimens (none at all in Human Biology) and are unsuited to the exhibition space provided by Waterhouse's building. Human Biology was built inside former galleries, which it conceals completely, and Ecology was set up in the former gallery of living mammals. (It has recently been moved to the Fossil Reptile Gallery, soon to be demolished, where it looks even more out of place.)

Yet for all these unpromising omens, the museum apparently does intend to show its collections. The "Evolution and diversity" exhibits will, it is stated, "contain most of the material now on show in the Museum", exemplifying 5,000 to 10,000 species. They "will continue to provide . . . the most entertaining aspect of the Museum, halls of monsters and a stuffed zoo." Now this is exactly what the Waterhouse galleries were designed to do. It seems the height of perversity not to use them for the purpose. On the contrary, it is proposed to dismantle the 1972 Fossil Mammals exhibit (an admirable exposition of evolution and diversity) and to demolish a large range of the original galleries. Surely it would be better to do the obvious thing, use the original galleries for displaying the collections, and put exhibits such as Human Biology in a communicating building outside the original structure.

It is clear that the authorities of the museum are, at best, embarrassed by the splendid interiors they have inherited. They do not know what to do with them, or how to make use of them constructively. At worst they appear to regard them merely as an impediment to be concealed and, if possible, removed.

Worse than this, there appears never to have been any coherent plan for the development of the buildings and the site as a whole. Both the original building, admirably suited to its original purpose but quite inadequate for research and storage, and the site are large, but not inexhaustible. For example, the new Palaeontology building (opened 1977) is splendid and fairly well suited for its purpose of research and storage, but it has used up the last available building plot next to the main building. This piecemeal approach exactly parallels the present attitude to the use of the interiors — exhibits and other facilities being

placed wherever there happens to be some space that can be occupied. The whole problem of space and buildings at the museum is in urgent need of review, preferably by an independent body since the Trustees seem to be unable to see the wood for the trees.

D. T. DONOVAN

Department of Geology,
University College London, UK

1. Halstead, L.B. *Nature* **288**, 208 (1980).
2. Halstead, L.B. *Nature* **275**, 683 (1978).
3. *Report on the British Museum (Natural History) 1972-74*, 75 (HMSO, London, 1975).

SIR—Halstead¹ has criticized the current exhibition policy of the British Museum (Natural History) on the grounds that the new dinosaur and fossil man exhibits are vehicles for the didactic presentation of a cladistic interpretation of evolution and taxonomy. He goes on to suggest that cladism and a saltationist interpretation of the history of life provide support for a Marxist view of history by progressive revolutions.

The political argument was questioned by Hughes-Games² and Rothman³ and is, really, a diversion from the main issue. Marxists and creationists may find support for their ideas in cladism and punctuated equilibria, but the interpretation placed on fact or hypothesis by people with a particular axe to grind should not affect the development of primary research.

Patterson⁴ presents some arguments on the validity of cladism and its relationships to evolutionary theory. This is an area that will probably continue to be discussed for many years (no doubt to the amazement and disgust of all non-participants).

However, the main issue is the exhibitions policy of the Museum. This question can be split into two: "Are the new exhibits balanced reflections of current scientific opinion?" and "Do the exhibits satisfy the public?"

The first point is answered with a resounding "no" by Halstead, and, I think, a partial "yes" by Patterson. Patterson states that "amongst scientists in the Museum there are many different viewpoints on the value and generality of cladistic methods", but this is not reflected in any exhibit yet. Is there any plan for a presentation of the views of "classical taxonomists" who regard cladism as an important analytical tool, but not as the sole aim and guiding principle of their research, or of numerical taxonomists (again with provisos as to applicability)?

The second question has not been asked yet. The new exhibition policy was started in the mid-1970s with the important aim of presenting a dynamic view of biological processes and it was stated that "... the scheme will be worthily carried out only if we can provide instruction and pleasure to the population as a whole ...".⁵ Is this aim satisfied?

I will comment only on the dinosaur exhibition. This occupies the prime gallery of the Museum — the main entrance hall. Large skeletons attract the visitor and strategically placed stalls market books, postcards, badges and models. The alcoves on either side contain the teaching parts of the exhibit and show examples of contemporaries of the dinosaurs and the much-criticized presentation of cladism. However, the interested visitor will search fruitlessly for information about life-style, function and physiology of dinosaurs. My experience from presenting lectures to school children, natural history societies and

adult education classes is that all these groups of laymen want to know if dinosaurs were warm-blooded, why they were so big, how they used their horns, spines and frills, how we collect them, why they died out, and so on. When I mention taxonomy there is a general glazing of eyes, fidgeting and covering of mouths. This may be an indictment of my teaching methods, but I think that it is also a reflection of what people consider interesting.

If the Public Services Department plans displays on dinosaur biology (functional anatomy, ecology, behaviour), I apologise for these remarks. If not, I don't.

MICHAEL J. BENTON

Department of Geology,
The University,
Newcastle upon Tyne, UK

1. Halstead, L.B. *Nature* **288**, 208 (1980).
2. Hughes-Games, M.J. *Nature* **288**, 430 (1980).
3. Rothman, H. *Nature* **288**, 430 (1980).
4. Patterson, C. *Nature* **288**, 430 (1980).
5. *Report of the British Museum of Natural History, 1972-74*, p.76 (Trustees of the British Museum (Natural History), 1975).

Halstead replies

SIR — In view of the seriousness of the criticisms¹ levelled against the Natural History Museum, it seems a little surprising that they failed to elicit any response from the Director or even the Head of the Public Services Department. The only letter from the museum was from a senior member of the Palaeontology Department², who appears to be concerned with roundly condemning the very policies which were the subject of my initial protest.

Patterson³ insists that "cladistics is not about evolution" and that there is "no connection between cladistics and one view of the evolutionary process". The "cladistic literature" to which he refers to support these assertions comprises only papers by Platnick⁴ and himself⁵, and these deal with what is known as "transformed cladistics". This is most definitely not the kind of cladistics being portrayed in the public galleries of the Natural History Museum, to which Patterson appears to be resolutely opposed. The museum's Public Services Department in fact accepts the classic version of cladistics as set out originally by Hennig in *Phylogenetic Systematics*⁶. The title of his book is frequently taken as a synonym of cladistics⁶, a term which refers specifically to the importance of the branching process (speciation) in phylogeny⁶. Hennig's view of the origin of new species involved the splitting of the ancestral species as a consequence of geographical isolation (allopatric speciation)⁶.

The long recognized (in spite of Patterson's contrary assertion²) connection between cladistics and punctuated equilibria, a fundamentally "leap" or saltation view of the process of evolution, has recently been emphasized by Cracraft⁷ and noted by others^{8,9}. Eldridge and Gould¹⁰ formulated the theory of "punctuated equilibria: an alternative to phyletic gradualism" by applying the concept of allopatric speciation, as used by Hennig, to the fossil record. In a subsequent review of the topic and the controversy it had engendered, Gould and Eldridge¹¹ contrasted the concept of gradualism "embedded in the modern history of Western cultures" with that of the "official 'state philosophy' of many socialist nations" where the "laws of change are explicitly punctuational", quoting Engels and the official Soviet Handbook of Marxism—

Leninism to this effect. Their basic Marxist approach to evolution has been generally recognized as such, by *inter alia* Gray¹² and Hughes-Games¹³. The latter and Lewin¹⁴ hold the view that the concept of punctuated equilibria is on the way to becoming the orthodoxy of the future. This being so, it is not unexpected that many of its new-found adherents may well be unaware of the philosophy behind the cause they are espousing.

The major controversy regarding the process of evolution and the fossil record concerns the notions of the origin of new species by sudden splitting (Hennig⁵, Eldridge and Gould^{10,11}) — the Marxist model, as against the gradualist model associated with Darwin¹⁵, Mayr¹⁶ and Simpson¹⁷. I am associated with the latter model, not from uncritically accepting the authority of Mayr and Simpson but on the basis of my own research experience.

The death of scholarship in the Natural History Museum is not in my opinion marked by the advocacy of cladistics or even Marxism in the public galleries, as Patterson² seems to imagine, but rather in the manner of the advocacy. The exhibits on dinosaurs and fossil man together with their accompanying books avoid any discussion of the gradual evolutionary versus the revolutionary "leap" concepts, by the simple expedient of accepting the basic assumptions of one and ignoring those of the other¹⁸. It is this crude partisanship which is indeed the unacceptable "strident voice of authority".

The insistence on a chosen received dogma, such as that no fossil species can be considered ancestral to any other, has led the Public Services Department into its obvious differences of opinion with the museum's Sub-Department of Anthropology over *Homo erectus*. That "there is not any serious doubt about *Homo erectus* being ancestral to *Homo sapiens*" is contrary to the rules of the cladistics as applied in the exhibit but is in fact the considered opinion of the scientific staff of the museum's own Sub-Department of Anthropology, and certainly the literature that immediately comes to hand appears to agree with this, including as it does cladistic analyses¹⁹⁻²⁴. The most telling evidence, however, comes from within the covers of the museum's own book, *Man's Place in Evolution*²⁵. I have already drawn attention¹, in this context, to the vitally important Petralona skull, the phylogenetic position of which was recently discussed in detail by Stringer, of the Natural History Museum¹⁹ and by others^{26,27}. The skull figures in the book, but curiously has been excluded from the exhibition itself. It would have served as a dramatic illustration of a transitional form exactly intermediate between *Homo erectus* and *Homo sapiens* but would at the same time have destroyed the credibility of the cladistic dogma being promoted. In the museum's public galleries, it now transpires that, unlike elsewhere in science, "ugly facts" are not to be permitted to "slay beautiful hypotheses".

The Director in his preface writes that, like the exhibit, the book "was planned with the guidance of Museum experts, particularly from the Museum's Sub-Department of Anthropology"²⁵. This gives the entirely misleading impression that what is on display and published by the museum represents the considered views of the museum's own anthropologists. This is most certainly not the case and one cannot help but wonder whether

the Director is actually aware of what is going on in the institution which he heads.

Finally, lest it be thought that my previous letter might be construed as "dangerous to the unfettered development of science"²⁸, in my estimation it is the current policies of the Public Services Department of the Natural History Museum that are already having just such an effect. I am not opposed to the public presentation of a reasoned case for cladistics, be it transformed or classical, as I have demonstrated by employing cladistic analysis in my own research²⁹; nor have I any objection whatsoever to the presentation of a Marxist interpretation of the history of life, if done in the open and scholarly manner of a Stephen Jay Gould.

My objection is to a major public scientific institution, renowned internationally for its scholarship, to be seen to be abusing its authority by attempting to impose on the general public, against the scientific judgement of its own experts, controversial concepts not by argument or discussion but simply by unsubstantiated assertion.

L. B. HALSTEAD

Departments of Geology and Zoology,
University of Reading, UK

1. Halstead, L. B. *Nature* **288**, 208 (1980).
2. Patterson, C. *Nature* **288**, 430 (1980).
3. Platnick, N. I. *Syst. Zool.* **26**, 438-442 (1977); **28**, 537-546 (1979).
4. Patterson, C. *Biologist* **27**, 234-240 (1980).
5. Hennig, W. *Phylogenetic Systematics* (Illinois University Press, Urbana, 1966, 1979).
6. Bonde, N. *Colloques int. Cent. natn. Rech. scient.* **218**, 283-324 (1975).
7. Cracraft, J. *Phylogenetic Analysis and Paleontology* (eds. Cracraft, J. & Eldridge, N.) (Columbia University Press, 1979).
8. Hull, D. L. *Syst. Zool.* **28**, 416-440 (1979); *Paleobiology* **6**, 131-136 (1980).
9. Fortey, R. *Pal. Ass. Circ.* **100**, 10-11 (1980).
10. Eldridge, N. & Gould, S. J. in *Models in Paleobiology* (ed. Schopf, T. J. M.) (Freeman, San Francisco, 1972).
11. Gould, S. J. & Eldridge, N. *Paleobiology* **3**, 225-151 (1977).
12. Gray, G. K. *New Scientist* **86**, 402 (1980).
13. Hughes-Games, M. J. *Nature* **288**, 430 (1980).
14. Lewin, R. *Science* **210** 883-887 (1980).
15. Darwin, C. *The Origin of Species by Means of Natural Selection or the Preservation of Favoured Races in the Struggle for Life* (Murray, London, 1859).
16. Mayr, E. *Systematics and the Origin of Species* (Columbia University Press, 1942); *Principles of Systematic Zoology* (McGraw-Hill, New York, 1969).
17. Simpson, G. G. *Tempo and Mode in Evolution* (Columbia University Press, 1944); *The Major Features of Evolution* (Columbia University Press, 1953); *Principles of Animal Taxonomy* (Columbia University Press, 1961).
18. Halstead, L. B. in *Marx Refuted* (eds Duncan, R. & Wilson, C.) (Pergamon, Oxford, 1981).
19. Stringer, C. *Anthropos* **7**, 81-93 (1980).
20. Bonde, N. *Major Patterns in Vertebrate Evolution* (eds Hecht, M. K., Goody, P. C. & Hecht, B. M.) (Plenum, New York, 1977).
21. Howell, F. C. in *Evolution of African Mammals* (eds Maglio, V. J. & Cooke, H. B. S.) (Harvard University Press, 1978).
22. Pilbeam, D. *The Evolution of Man* (Thames & Hudson, London, 1970).
23. Washburn, S. L. *Scient. Am.* **239**, 146-154 (1978).
24. Wood, B. *The Evolution of Early Man* (Peter Lowe, London, 1976).
25. *Man's Place in Evolution* (British Museum (Natural History) London, 1980).
26. Jelinek, F. in *Current Arguments on Early Man* (ed. Königsson, L. -K.) (Pergamon, Oxford, 1980).
27. Wolpoff, M. H. *J. hum. Evol.* **2**, 339-358 (1980).
28. Rothman, H. *Nature* **288**, 430 (1980).
29. Halstead, L. B. in *Problems of Phylogenetic Reconstruction* (eds Joysey, K. A. & Friday, A. E.) (Academic, London, 1981).

Science and values

SIR—Between November 27 and 30 1980 I attended, as an observer, the 9th International Conference on the Unity of Sciences (ICUS) in Miami Beach. It was host to 640 participants from 85 countries. The consensus of the overwhelming majority of those who attended

was that the conference, under the banner "Absolute Values and the Search for the Peace of Mankind", was a great success. Imagine, then, the consternation with which I read your editorial comment (*Nature* 27 November p.310) on ICUS "Best not to attend on Mr Moon", which launched scathing broadsides on the founder, seemingly, the aims of the conference, and, indirectly on the integrity of the participants themselves.

Science and values, naturally, reside in different worlds. One cannot understand man's spiritual quest (for liberty, truth, beauty etc.) by empirical laws nor can one restrict scientific endeavour with religious dogma. Yet who can pretend, in a world exhibiting both desperate physical need and social disintegration, that they have nothing to do with each other? This is where I consider the ICUS makes its unique contribution — as an interdisciplinary forum where scholars can freely exchange ideas developed in their own fields about the pressing needs of the world. Few would consider this an "extraneous cause". Are we to understand then, that in referring to Moon's addresses as "vacuous", and implying the term "values" to be meaningless, the editorial policy of *Nature* is to preserve the steps up the ivory tower of scientific learning unsullied by the muddy boots of morality and social responsibility?

Another point of contention is the assertion that "the participants . . . are (not) the group ideally suited for the discussion of the broad themes . . .". If eminent academics in the fields of science, philosophy and the humanities are *not*, then who is?

The conclusion of one committee chairman at the end of the Miami conference was that probably more questions had been raised than answered. I would agree with this. The aims of a body like the ICUS are not easily achieved but the challenge to the academic community to work towards the unity of the sciences can only have positive results, more than satisfying merely an intellectual appetite.

D.M. TRUBSHAW

London W2, UK

NSF and cryptology

SIR — In view of the extensive recent discussion of the respective roles of the National Science Foundation (NSF) and the National Security Agency (NSA) in support of cryptological research (see, for example, *Nature* 4 September, p.2), I believe it may be useful to restate the foundation's established policy in this area.

The essential points of our policy with respect to cryptological research are these:

(1) Since mid-1977 we have routinely referred proposals with relevance to cryptology to NSA for review. We will continue to do this. The practice serves to keep NSA informed of NSF's activities in this area, and gives NSA an opportunity to make technical comments on proposals which can be useful in making funding decisions. It is not a "clearance" process; whatever comments NSA may make are advisory.

(2) NSF has long had a policy of encouraging other agencies to support basic research in areas relevant to their missions. We have specifically encouraged NSA to establish an unclassified basic research programme, and stand ready to assist that agency in this effort. We believe it is fundamentally healthy to have alternative sources of support in important areas of science, and anticipate no difficulties

in maintaining close coordination between NSF and NSA.

(3) In cases in which alternative sources of support are available, we routinely encourage principal investigators to apply to such sources as well as to NSF. However, if an investigator prefers to apply only to NSF, we will consider the proposal in the usual manner, without prejudice, and reach a decision on funding using our usual criteria and peer review process.

(4) NSF does not expect that the results of the basic research which it supports will be classified, except in very rare instances. NSF does not currently have classification authority, but it has responsibility, under routine executive orders issued by both the current and previous administrations, to refer any information which it believes might require classification to the agency with appropriate subject matter interest and original classification authority. For cryptological research, that agency is NSA. The important point here is that it makes no essential difference, in terms of the likelihood of classification, whether research is supported by NSF or NSA. This policy is of long standing, and applies to all areas of research.

(5) NSF has long-established reporting requirements which allow it to meet its responsibility for prudent use of public funds. These might not be adequate in all cases where research might have special relevance to national security, and in such cases we may consider special reporting requirements. We have not done this in the past, and we may not have to do it in the future. If we did have to establish such reporting requirements, however, we would regard this not as a change in policy but simply as a change in administrative procedure necessary to apply a long-standing policy to a changed situation.

In summary, the foundation will continue to support cryptological research, will continue to coordinate such research with NSA, and will continue to encourage NSA to develop its own basic research support programme. The results of such research have not been classified in the past, and we do not expect them to be in the future, but we will ensure that our reporting requirements are adequate to allow us to meet our responsibilities with respect to possible classification. Most importantly, the foundation has a basic policy of supporting the best research it can find in all areas of science and engineering, with the fewest possible restrictions on investigators.

DONALD N. LANGENBERG
(Acting Director)

National Science Foundation,
Washington, DC

Behind the fridge

SIR—It is highly likely that the reservoir at the back of a self-defrosting refrigerator provides an excellent breeding ground for microorganisms (*Nature* 20 November 1980, p. 208). However, your correspondent's suggestion that these organisms might cause pulmonary infections such as Legionnaires' disease neglects to consider that they would first have to be dispersed in the form of an aerosol. It is difficult to imagine how this might happen other than when the machine is subject to violent movement.

P.A. JENKINS
Public Health Laboratory Service,
Mycobacterium Reference Unit, Cardiff, UK

Old *Nature's* almanac for 1981

In early times the beginning of each year was marked by the publication of almanacs intended to chronicle events in the succeeding months. Most of these documents were necessarily blends of fact and fiction. The following, however, is an authentic chronicle of the new year.

January: Bitter weather. President Ronald Reagan (20 January) says at inauguration that "we have nothing to hope for but hope itself", and commits the United States to "making the shuttle work within this decade". Dr William Nirenberg appointed chairman of reconstituted President's Science Advisory Committee. Yugoslavia establishes biotechnology enterprise (capital \$20 million) with licence to manufacture insulin from "porcine pancreatic tissue". *Nature* publishes 25-page human mitochondrial DNA sequence. Earthquakes in Tashkent. Mercury and Mars ill-placed for observation.

February: Bitter weather (except in Southern Hemisphere). General Alexander Haig reaffirms opposition to Salt II at Senate confirmation hearings on nomination as US Secretary of State. Mass of neutrino measured at Savannah River reactor experiment as 30 ± 35 electron volts; US Department of Energy announces "riddle of Universe all but solved" and President Reagan gives new go-ahead for Clinch River fast reactor. *Nature* publishes first instalment of DNA sequence of human chromosome 7 (F. Sanger); Unesco announces "emergency meeting" of journals editors (Addis Ababa, 1985) to consider "sequence pollution". Interim report of Swinnerton-Dyer committee on organization of University of London says that university must save money "or face prospect of radical change". Earthquakes in Hellenic arc. Venus and Mars ill-placed for observation.

March: Bitter weather; much flood. American journal *Science* begins publication of nucleotide sequence of human chromosome 7 (W. Gilbert); Senator William Proxmire announces public hearings on question "Are sequences necessary?". US General Accounting Office recommends that Shuttle Tile Repair Module be adapted as skyscraper window-cleaning machine. Neutrino mass estimated as 50 ± 55 electron volts by observation of diffuse ultraviolet background; NASA announces "mystery of Universe now cleared up". British universities told that in future, *all* students must pay economic fees. General Alexander Haig (at continued Senate hearing) reaffirms opposition to Comprehensive Test-Ban Treaty. Earthquakes in Caribbean. Mercury, Venus and Mars ill-placed for observation.

April: Sakharov writes to announce arrival of Spring in Gor'kii. *Nature* begins publishing errata to Sanger chromosome 7 sequence. Tanzanian Secretary-General of Addis Ababa conference on sequences calls for extension of terms of reference to include measurement of neutrino mass, and charges that present practices of journal editors discriminate against developing nations. First shuttle flight crew sues NASA over early retirement terms. British Chancellor of Exchequer levies Value Added Tax on university fees, but affirms "monetarism is working". Professor Milton Friedman appointed Companion of Honour. Earthquakes in the Levant. Mercury, Venus and Mars ill-placed for observation.

May: Unseasonable weather. Soviet government accedes to Sakharov's request to be put on trial, but postpones formal charges. Dr Edward Parkes (chairman, UK University Grants Committee) says "no compelling reason" why British universities should be closed, but confesses anxiety about recurrent grant. Centenary celebrations of British Museum (Natural History) pass off without incident; cladists and opponents silenced by Royal presence. Dr F. Sanger forswears nucleotide sequencing until "somebody decides what to do with the data". President Reagan, by executive order, merges Clinch River and shuttle projects, offering management contract to Government of France. General Haig tells Senate confirmation committee that US Administration will "seek to repeal" Anti-Proliferation Act. Earthquakes in Andes. Mars ill-placed for observation.

June: Bitter weather in Antarctica; much flood elsewhere. Morrison committee on "dual-support system" for UK academic research argues that because academic research has continued even though the system has "long since" broken down, there is no special need of new mechanisms for research support. Proxmire Senate committee condemns sequencing research as "pork-barrel biochemistry" and proposes legislation denying federal funds to institutions defining sequences of "more than 52 contiguous nucleotides". General Haig confirmed as US Secretary of

State after promising to press for early signature of Comprehensive Test-Ban Treaty and ratification of "upgraded Salt II". Rockwell International (main shuttle contractor) announces development of Shuttle Tile Repair Kit as personal space transport module. Earthquakes in Italy. Mercury and Mars ill-placed for observation.

July: Inclement weather. House of Lords committee on "Science and Government" urges reorganization of UK science with separation of research councils from Department of Education, appointment of minister with responsibility for science and re-creation of Advisory Council on Scientific Policy. UK biotechnology company Celltech takes licence on Yugoslav insulin process. Committee of Vice-Chancellors and Principals announces "agreement in principle" with British Tourist Board that universities will put facilities and staffs at disposal of tourist industry during future extended long vacations in exchange for commission on tourist receipts; growing anxiety about future of Salford and Aston universities. NIH reaches compromise with Proxmire committee on safeguards procedure for regulation of sequencing work. Eclipse of Sun (31 July) visible in Middle East and central Asia. Mercury ill-placed for observation.

August: Improbable weather. NASA commissions Boeing design of reusable long-distance transport system based on 747 with "orbital operation development capability". US Department of Energy dedicates Clinch River site as "national center" for measurement of neutrino mass. Second Voyager approaches Saturn; Jet Propulsion Laboratory announces that "mankind has learned more about the Solar System in the past month than in the previous twelve". *Nature* finishes publishing errata to Sanger chromosome 7 sequence (see April). Europe on vacation. Mercury, Mars and Saturn ill-placed for observation.

September: Bitter weather. Sakharov trial postponed. British universities postpone *sine die* opening of new academic year in plan to avoid shutdown. UK government abolishes posts of chief scientist in government departments, but denies abandonment of Rothschild "customer-contractor" principle, pleading only "further steps in battle against public expenditure". Legality of NIH Advisory Committee on Nucleotide Sequences tested in US Supreme Court; Senator Proxmire agrees to modify bill for fear that "United States will lose out to foreign sequencers". Neutrino mass held "indistinguishable from zero" on basis of tritium decay measurement; Clinch River site re-dedicated as "National Nucleotide Sequence Repository". Mercury, Jupiter and Saturn ill-placed for observation.

October: Bitter weather. Interim shuttle modification programme approved by NASA; US General Accounting Office complains that the Orbitwise Re-entry Module Assembly Kit will leave no room in the cargo bay for payload, and that the decision that the shuttle vehicle itself should be disposable will have "negative economic consequences except in California". UK research councils say in annual reports that they have survived better than feared in worsening economic climate and that abandonment of grant-making is only temporary. Dr J. L. Gowans, Secretary of Medical Research Council, denies at press conference statement attributed to him that "medical research is mostly good luck" (see *Nature* 1/8 January, p.2); rather, says Gowans, "inspired guesswork". Mercury, Jupiter and Saturn ill-placed for observations.

November: Extremes of climate. Nobel prizes: Medicine, Dr César Milstein (for monoclonal antibodies); Chemistry, Dr Fred Sanger (for a method of sequencing carbohydrate macromolecules); Physics, Professor F. W. Reines (for drawing attention to the problem of the neutrino mass). NIH announce further relaxation of sequencing guidelines; joint NIH-EMBO sequence bank commissions advanced computer design from Rockwell International. No planets ill-placed for observation.

December: Weather mixed. Swinnerton-Dyer report on organization of University of London offers "stark" choice between expulsion of and management by Imperial College; university Senate resolves that changes of function unwelcome to those affected should not be effected. Vice-chancellors announce British universities will begin academic year once cold weather is ended (to avoid excessive heating bills) and "certainly no later than beginning of tourist season". Boeing claims "synchronous orbit capability" for 747; proposes development programme for returning flying machines to Earth. Proxmire committee begins investigation of "neutrino mass boondoggle". Harvard University converts P3 laboratory to squash-court. Earthquakes in Tashkent. Mercury again ill-placed for observation.